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**REGISTERED PATENT AGENTS

June 16, 1999

WRITER'S DIRECT NUMBER: (202) 789-5509

Assistant Commissioner for Patents
Washington, D.C. 20231

Box: Patent Application

Re: U.S. Non-Provisional Utility Patent Application under 37 C.F.R. § 1.53(b)
Appl. No. To be assigned; Filed: Herewith
(Divisional of Appl. No. 08/815,469; Filed: March 11, 1997)
For: **Death Domain Containing Receptors**
Inventors: Yu *et al.*
Our Ref: 1488.0310005/EKS/SGW

Sir:

The following documents are forwarded herewith for appropriate action by the U.S.
Patent and Trademark Office:

1. Fee Transmittal Form (PTO/SB/17) (in duplicate);
2. USPTO Utility Patent Application Transmittal Form PTO/SB/05;
3. 37 C.F.R. § 1.136(a)(3) Authorization to Treat a Reply As Incorporating An Extension of Time (in duplicate);
4. U.S. Utility Patent Application entitled:

Death Domain Containing Receptors

Assistant Commissioner for Patents
June 16, 1999
Page 2

and naming as inventors:

Guo-Liang Yu,
Jian Ni,
Reiner L. Gentz, and
Patrick J. Dillon

the application comprising:

(a) A specification containing:

- (i) 89 pages of description prior to the claims;
- (ii) 7 pages of claims (Claims 1-26);
- (iii) a one (1) page abstract;

(b) 6 sheets of drawings: (Figures 1A, 1B, 2, 3, and 4);

5. Combined Declaration and Power of Attorney for Patent Application executed by Guo-Liang Yu, Jian Ni, Reiner L. Gentz, and Patrick J. Dillon as filed in parent Appl. No. 08/815,469;
6. Combined Declaration and Power of Attorney for Patent Application executed by Vishva Dixit as filed in parent Appl. No. 08/815,469;
7. Request to Open New Disk File;
8. First Preliminary Amendment;
9. Second Preliminary Amendment with Exhibits A and B;
10. ATCC Deposit Receipt for Deposit No. 97456;
11. ATCC Deposit Receipt for Deposit No. 97757;
12. Our Check No. 24665 in the amount of \$838.00 to cover:

\$760.00	Application filing fee;
\$ 78.00	Independent claims in excess of three fee; and

Assistant Commissioner for Patents

June 16, 1999

Page 3

13. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Stephen A. Hulse

Stephen G. Whiteside
Attorney for Applicants
Registration No. 42,224

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SKGF Rec 9/22/98dcw

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PTO/SB/05 (2/98)

Approved for use through 09/30/2000. OMB 0651-0032
Patent and Trademark Office U.S. DEPARTMENT OF COMMERCE

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UTILITY PATENT APPLICATION TRANSMITTAL <i>(Only for new nonprovisional applications under 37 CFR § 1.53(b))</i>	Attorney Docket No.	1488.0310005/EKS/SGW
	First Inventor or Application Identifier	Guo-Liang Yu
	Title	Death Domain Containing Receptors
	Express Mail Label No.	

APPLICATION ELEMENTS <i>See MPEP chapter 600 concerning utility patent application contents</i>	ADDRESS TO: Assistant Commissioner for Patents Box Patent Application Washington, DC 20231
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1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 97]
(preferred arrangement set forth below)
- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 6]
4. ☒ Oath or Declaration [Total Pages 6]
a. ☐ Newly executed (original or copy)
b. ☒ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17 completed)*
[Note Box 5 below]
i. ☒ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR §§ 1.63(d)(2) and 1.33(b). (See Second Preliminary Amendment.)
5. ☒ Incorporation By Reference *(useable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein
6. ☐ Microfiche Computer Program *(Appendix)*
7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
a. ☐ Computer Readable Copy
b. ☐ Paper Copy (identical to computer copy)
c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☒ First Preliminary Amendment
Second Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ *Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired
(PTO/SB/09-12)
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ☒ Other 37 C.F.R. § 1.136(a)(3) Authorization (in duplicate)
☒ Other: Request to Open New Disk File
- *NOTE FOR ITEMS 1 & 14 IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28)

17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment.

☐ Continuation ☒ Divisional ☐ Continuation-in-Part (CIP) of prior application No: 08/815,469

Prior application information: Examiner Ulm, J. Group/Art Unit: 1646

18. CORRESPONDENCE ADDRESS

☐ Customer Number
or Bar Code Label

(Insert Customer No. or Attach bar code label here)

or ☒ Correspondence
address below

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	Attorneys at Law				
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NAME (Print/Type)	Stephen G. Whiteside	Registration No. (Attorney/Agent)	42,224
SIGNATURE		Date	6/16/99

Burden Hour Statement: this form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

YU *et al.*

Appl. No. To be assigned
(Divisional of Appl. No.
08/815,469, Filed: March 11, 1997)

Filed: Herewith

For: **Death Domain Containing
Receptors**

Art Unit: To be assigned

Examiner: To be assigned

Atty. Dkt.: 1488.0310005/EKS/SGW

First Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In advance of prosecution, please amend the captioned application as follows:

In the Claims:

Please cancel claims 1-21 and 23-26 without prejudice of or disclaimer to the subject matter therein.

Remarks

I. Status of the Claims

Claims 1-21 and 23-26 have been canceled. Accordingly, claim 22 is active in the present application. No new matter has been added by this amendment.

A Second Preliminary Amendment is being filed herewith. In the Second Preliminary Amendment, claim 22 is canceled and new claims 27-46 are added.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Stephen G. Whiteside
Attorney for Applicants
Registration No. 42,224

Date:

1100 New York Avenue, N.W.
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

YU *et al.*

Appl. No. To be assigned
(Divisional of Appl. No.
08/815,469, Filed: March 11, 1997)

Filed: Herewith

For: **Death Domain Containing
Receptors**

Art Unit: To be assigned

Examiner: To be assigned

Atty. Dkt.: 1488.0310005/EKS/SGW

Second Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

It is respectfully requested that the following amendments to the specification and claims be entered in advance of substantive examination.

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

In the Inventorship:

Please delete "Vishva Dixit" as an inventor.

In the Specification:

Please amend the specification as follows:

On page 1, lines 3-7, please delete the Cross-Reference to Related Applications section and replace it with the following:

--The present application is a divisional of U.S. Application No. 08/815,469, filed March 11, 1997, which is herein incorporated by reference; said 08/815,469 claims priority to U.S. Provisional Application No. 60/013,285, filed March 12, 1996, U.S. Provisional Application No. 60/028,711, filed October 17, 1996, and U.S. Provisional Application No. 60/037,341, filed February 6, 1997, each of which is herein incorporated by reference.--

At page 5, line 1, please delete "in a bacterial host".

On page 7, line 26, please delete "shares" and substitute therefor --share--.

On page 8, lines 3-4, please delete "12301 Park Lawn Drive, Rockville, Maryland 20852" and insert therefor --10801 University Blvd., Manassas, VA 20110-2209, USA--.

On page 8, lines 8-9, please delete "12301 Park Lawn Drive, Rockville, Maryland 20852" and insert therefor --10801 University Blvd., Manassas, VA 20110-2209, USA--.

On page 10, line 18, after "species" please delete "on" and substitute therefor --of--.

On page 13, line 27, please delete "hybridiz" and substitute therefor --hybridization with chromosomes, and for detecting expression of the--.

On page 14, line 13, please delete "DR3" and substitute therefor --DR3-V1--;

On page 14, line 15, please delete "DR3" and substitute therefor --DR3-V1--;

On page 14, line 17, please delete "DR3" and substitute therefor --DR3-V1--;

On page 14, line 17, please delete "214" and substitute therefor --236--; and

On page 14, line 18, please delete "DR3" and substitute therefor --DR3-V1--.

At page 14, between lines 23 and 24, please insert the following paragraph:

--Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding: amino acid residues from about 1 to about 215 of SEQ ID NO:2; amino acid residues from about 30 to about 215 of SEQ ID NO:2; amino acid residues from about 215 to about 240 of SEQ ID NO:2; amino acid residues from about 240 to about 428 of SEQ ID NO:2; and amino acid residues from about 350 to about 420 of SEQ ID NO:2.--

On page 15, line 24, please delete "(150mM NaCl, 15mM trisodium citrate)" and insert --(750mM NaCl, 75mM trisodium citrate)--.

On page 15, line 25, please delete "20 g/ml" and insert therefor --20 µg/ml--.

On page 23, line 19, please delete "the is" and substitute therefor --is then--.

On page 33, line 5, after "such" and before "the", please insert --as--.

At page 33, between lines 24 and 25, please insert the following paragraph:

--Preferred polypeptide fragments of the present invention also include: amino acid residues from about 1 to about 215 of SEQ ID NO:2; amino acid residues from about 30 to about 215 of SEQ ID NO:2; amino acid residues from about 215 to about 240 of SEQ ID NO:2; amino acid residues from about 240 to about 428 of SEQ ID NO:2; and amino acid residues from about 350 to about 420 of SEQ ID NO:2.--

On page 61, line 23, please delete "5 gCi" and insert therefor --5 µCi--.

On page 63, line 1, please delete "(SEQ ID NO:1)" and substitute therefor --(SEQ ID NO:3)--.

On page 63, line 24, please delete "(Rockville, MD)" and insert therefor --(Manassas, VA)--.

On page 64, line 16, please delete "etidium" and insert therefor --ethidium--.

069496 : 06499

In the Claims:

Please cancel claim 22 without prejudice or disclaimer of the subject matter therein.

Please add the following new claims:

- 27. An isolated protein comprising amino acids 30 to 215 of SEQ ID NO:2.
28. The isolated protein of claim 27, which comprises amino acids 1 to 215 of SEQ ID NO:2.
29. The isolated protein of claim 27, which is produced by a recombinant host cell.
30. The isolated protein of claim 27, which comprises a heterologous polypeptide.
31. A composition comprising the isolated protein of claim 27 and a pharmaceutically acceptable carrier.
32. An isolated protein comprising the mature amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.
33. The isolated protein of claim 32, which comprises the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.
34. The isolated protein of claim 32, which is produced by a recombinant host cell.

35. The isolated protein of claim 32, which comprises a heterologous polypeptide.
36. A composition comprising the isolated protein of claim 32 and a pharmaceutically acceptable carrier.
37. An isolated protein comprising 30 contiguous amino acids of a polypeptide having the amino acid sequence from 350 to 420 of SEQ ID NO:2.
38. The isolated protein of claim 37, which comprises 50 contiguous amino acids of a polypeptide having the amino acid sequence from 350 to 420 of SEQ ID NO:2.
39. The isolated protein of claim 38, which comprises amino acids 350 to 420 of SEQ ID NO:2.
40. The isolated protein of claim 37, which is produced by a recombinant host cell.
41. The isolated protein of claim 37, which comprises a heterologous polypeptide.
42. A composition comprising the isolated protein of claim 37 and a pharmaceutically acceptable carrier.
43. An isolated protein comprising amino acid residues encoded by a first polynucleotide which hybridizes to a second polynucleotide consisting of nucleotides 1245 to 1457 of SEQ ID NO:1, or the complement thereof, under the following conditions:

- (a) incubating overnight at 42°C in a solution consisting of 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA; and
- (b) washing at 65°C in a solution consisting of 0.1x SSC.

- 44. The isolated protein of claim 43, which is produced by a recombinant host cell.
- 45. The isolated protein of claim 43, which comprises a heterologous polypeptide.
- 46. A composition comprising the isolated protein of claim 43 and a pharmaceutically acceptable carrier.--

Remarks

After cancellation of claim 22 and entry of the claims set out above, claims 27-46 will be pending in the captioned application, with claims 27, 32, 37, and 43 being the independent claims.

I. The Priority Applications

The captioned application claims priority benefit of U.S. Application No. 08/815,469, filed March 11, 1997; U.S. Provisional Application No. 60/013,285, filed March 12, 1996; U.S. Provisional Application No. 60/028,711, filed October 17, 1996; and U.S. Provisional Application No. 60/037,341, filed February 6, 1997. Each of these applications have been incorporated by reference into the captioned application. (Specification, page 1, lines 3-7.)

II. Amendment to the Inventive Entity

Deletion of Vishva Dixit from the inventive entity is respectfully requested, since Guo-Liang Yu, Jian Ni, Reiner L. Gentz, and Patrick J. Dillon are the inventors of the currently claimed invention. The amendment set out above is thus required so that the inventive entity includes only the correct inventors.

III. The Amendments to the Specification

The specification has been amended to include the new address of the American Type Culture Collection (ATCC). The ATCC has recently moved from 12301 Park Lawn Drive, Rockville, MD 20852 to 10801 University Blvd., Manassas, VA 20110-2209. Applicants were advised on May 19, 1998, in a notification published in the Official Gazette, to amend pending applications to refer to the current address of the ATCC. (1210 OFF. GAZ. PAT. OFFICE 74 (May 19, 1998).) The amendments to page 8, lines 3-4 and 8-9, and page 63, line 24, are required to incorporate the new address of the ATCC into the specification. No new matter has been added by these amendments.

The specification of the captioned application has been amended to introduce subject matter from the '285 priority application. Since the '285 application was incorporated by reference into the captioned application at the time of filing (*see* specification, page 1, lines 3-7), the introduction of this subject matter does not constitute new matter.

In particular, the specification has been amended to include the following polypeptide fragments of SEQ ID NO:2, and polynucleotides which encode these fragments: amino acid residues from about 1 to about 215 of SEQ ID NO:2; amino acid residues from about 30 to about 215 of SEQ ID NO:2; amino acid residues from about 215 to about 240 of SEQ ID NO:2; amino

acid residues from about 240 to about 428 of SEQ ID NO:2; and amino acid residues from about 350 to about 420 of SEQ ID NO:2. For support, the Examiner is directed to the '285 priority application at page 11, lines 9-19, and page 30, lines 20-30. Accordingly, no new matter has been added by this amendment.

The specification has also been amended to correct an error in the form of the biological materials deposited as ATCC Deposit Nos. 97456 and 97757. In particular, page 5, line 1, of the specification states that the cDNA clones encoding the DR3-V1 (ATCC Deposit No. 75456) and DR3 (ATCC Deposit No. 97757) polypeptides were deposited in bacterial hosts. However, as evidenced by the attached ATCC deposit receipts, these cDNAs were deposited as plasmid DNAs.

Page 15, line 24, of the specification has been amended to correct an obvious typographical error in the amount of ingredients listed for 5x SSC (sodium chloride/sodium citrate). An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of the error in the specification, but also the appropriate correction. (M.P.E.P. § 2163.07.) Here, the recognition of the typographical error, along with the correction of the error, in the amount of the ingredients listed for 5x SSC, is obvious to one skilled in the art, and, therefore, the correction does not constitute new matter.

5x SSC is a well known solution used in hybridization solutions. (*See, e.g.,* Exhibit A, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons, N.Y., Supplement 35, page 2.10.7 (1996).) SSC is normally made as a 20x stock solution, and then diluted accordingly for a particular use. Exhibit B shows that a 20x SSC stock solution contains 3M NaCl and 0.3M trisodium citrate. (Exhibit B, CURRENT PROTOCOLS, page A.2.5.) To make a 5x SSC solution, the 20x SSC solution must be diluted by one-fourth. Therefore, a 5x SSC solution contains 750mM NaCl ($3\text{M} \div 4 = 750\text{mM}$) and 75mM trisodium citrate ($0.3\text{M} \div 4 = 75\text{mM}$).

One skilled in the art would have immediately recognized that the amount of ingredients listed in the specification for a 5x SSC solution was incorrect. Rather than describing a 5x SSC solution, made up of 750mM NaCl and 75mM trisodium citrate, the specification inaccurately listed the ingredients for a 1x SSC solution. Further, the skilled artisan, in recognizing the typographical error, could easily have adjusted the amount of ingredients described in the specification to properly make a 5x SSC solution. Therefore, the correction of this typographical error does not introduce new matter.

The amendment to page 15, line 25, of the specification, from "20 g/ml" to "20 µg/ml", is supported by page 13, lines 23-24, of U.S. Provisional Application Serial No. 60/013,285, filed March 12, 1996. As noted above, U.S. Provisional Application Serial No. 60/013,285 has been incorporated by reference into the captioned application. (Specification, page 1, lines 3-7.) Thus, the amendment of page 15, line 25, does not introduce new matter.

The specification has also been amended to correct errors of a typographical nature. Support for the amendment to page 13, line 27, can be found in the specification, at page 23, line 10, to page 24, line 16, page 19, line 28, to page 20, line 4, and pages 62, line 1, to page 65, line 12 (Example 4). Support for the amendment to page 14, lines 13-18, can be found in the specification, at page 7, lines 2-6, and page 33, lines 14-24. Support for the amendment to page 63, line 1, can be found in the specification, at page 10, lines 3-5. No new matter has been added by these amendments.

IV. Claims 27-46 are Supported by the Specification of the '285 Application

Support for new claims 27-46 can be found throughout the '285 application. In particular, support for claims 27-28, 32-33, and 37-39 can be found in the '285 application, *inter alia*, in claims 1, 6, and 20-25; in SEQ ID NOs:1 and 2; at page 7, line 33, to page 8, line 3; at page 8,

lines 14-24; and at page 30, lines 2-13. Support for claims 29-31, 34-36, 40-42, and 44-46 can be found in the '285 application, *inter alia*, at page 24, line 1, to page 28, line 28, and page 44, line 8, to page 46, line 33. Support for claim 43 can be found in the '285 application, *inter alia*, at page 7, line 33, to page 8, line 3, and at page 13, lines 17-25.¹

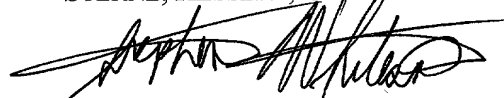
Conclusion

It is respectfully believed that this application is now in condition for substantive examination. Early notice to this effect is respectfully requested.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Stephen G. Whiteside
Attorney for Applicants
Registration No. 42,224

Date: 6/16/99

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P91-64.wpd

¹Applicants note that nucleotides 1245 to 1457 of SEQ ID NO:1 of the captioned application are identical to nucleotides 1245 to 1457 of SEQ ID NO:1 of the '285 application. Further, these nucleotides encode amino acids 350 to 420 of SEQ ID NO:2.

003306 0469

Death Domain Containing Receptors

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/013,285, filed March 12, 1996, U.S. Provisional Application No. 60/028,711, filed October 17, 1996, and U.S. Provisional Application No., to be assigned, filed February 6, 1997. The content of each of these applications is incorporated by reference in their entirety.

Background of the Invention

Field of the Invention

The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptors (DR3 and DR3-V1). Death Domain Containing Receptor polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR3 activity.

Related Art

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intracellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory

disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

5 Among the ligands there are included TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40L, CD27L, CD30L, 4-1BBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 10 4-1BB, OX40, low affinity p75 and NGF-receptor (A. Meager, *Biologicals*, 22:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (A. Meager, *supra*).

15 Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (R. Watanabe-Fukunaga *et al.*, *Nature* 356:314 (1992)), perhaps reflecting 20 a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (R.C. Allen *et al.*, *Science* 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by 25 faulty sensory innervation of peripheral structures (K.F.Lee *et al.*, *Cell* 69:737 (1992)).

TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted

tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (B. Beutler and C. Von Huffel, *Science* 264:667-668 (1994)). Mutations in the p55 receptor cause increased susceptibility to microbial infection.

Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for transducing signals for programmed cell death (Tartaglia *et al.*, *Cell* 74:845 (1993)).

Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland *et al.*, *Cell* 81, 479-482 (1995); A. Fraser *et al.*, *Cell* 85, 781-784 (1996); S. Nagata *et al.*, *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith *et al.*, *Science* 248, 1019-23 (1990); M. Tewari *et al.*, in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain," which is distantly related to the *Drosophila* suicide gene, reaper (P. Golstein *et al.*, *Cell* 81, 185-6 (1995); K. White *et al.*, *Science* 264, 677-83 (1994)). This shared death domain suggests

that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan *et al.*, *Cell* 81, 505-12 (1995); M. P. Boldin *et al.*, *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin *et al.*, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia *et al.*, *Immunol Today* 13, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu *et al.*, *Cell* 81, 495-504 (1995); H. Hsu *et al.*, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation, *Id.*; H. Hsu *et al.*, *Immunity* 4, 387-396 (1996)).

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize novel members of the TNF receptor family.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising nucleic acid sequences encoding the amino acid sequences shown in FIG. 1 (SEQ ID NO:2) and FIG. 2 (SEQ ID NO:4) or the amino acid sequence

encoding the cDNA clones deposited in a bacterial host as ATCC Deposit No. 97456 on March 1, 1996 and ATCC Deposit No. 97757 on October 10, 1996.

The present invention also provides vectors and host cells for recombinant expression of the nucleic acid molecules described herein, as well as to methods of making such vectors and host cells and for using them for production of DR3 or DR3 Variant 1 (DR3-V1) (formerly named DDCR) polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DR3 or DR3-V1 polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR3 or DR3-V1 protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR3 or DR3-V1, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. Cellular response to TNF-family ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3 polypeptide an effective amount of an agonist capable of increasing DR3 mediated signaling. Preferably, DR3 mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3 polypeptide an effective amount of an antagonist capable of decreasing DR3 mediated signaling. Preferably, DR3 mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR3 or DR3-V1 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DR3 or DR3-V1 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

FIG. 1A-B shows the nucleotide and deduced amino acid sequence of DR3-V1. It is predicted that amino acids 1 - 35 constitute the signal peptide, amino acids 36-212 constitute the extracellular domain, amino acids 213-235 constitute the transmembrane domain, amino acids 236-428 constitute the intracellular domain, and amino acids 353-419 the death domain.

FIG. 2 shows the nucleotide and deduced amino acid sequence of DR3. It is predicted that amino acids 1 - 24 constitute the signal peptide, amino acids 25-201 constitute the extracellular domain, amino acids 202-224 constitute the transmembrane domain, amino acids 225-417 constitute the intracellular domain, and amino acids 342-408 constitute the death domain.

FIG. 3 shows the regions of similarity between the amino acid sequences of the DR3-V1, human tumor necrosis factor receptor 1, and Fas receptor [SEQ ID NOs:5 and 6].

FIG. 4 shows an analysis of the DR3-V1 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues 1-22, 33-56, 59-82, 95-112, 122-133, 161-177, 179-190, 196-205 in Figure 1 correspond to the shown highly antigenic regions of the DR3-V1 protein.

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising a nucleic acid sequence encoding the DR3-V1 or DR3 polypeptide whose amino acid sequence is shown in FIG. 1 [SEQ ID NO:2] and FIG. 2 [SEQ ID NO:4], respectively, or a fragment of the polypeptide. The DR3-V1 and DR3 polypeptides of the present invention shares sequence homology with human TNF

RI and Fas (FIG. 4). The nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] was obtained by sequencing the HTTNB61 clone, which was deposited on March 1, 1996 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97456. The deposited clone is contained in the pBluescript SK(-) plasmid (Stratagene, LaJolla, CA). The nucleotide sequence shown in FIG. 2 [SEQ ID NO:3] was obtained by sequencing a clone obtained from a HUVEC library, which was deposited on October 10, 1996 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97757. The deposited clone is contained in the pBluescript SK(-) plasmid (Stratagene, LaJolla, CA).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence

actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposes of the invention, as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, *Gene* 67:31-40 (1988).

Using the information provided herein, such as the nucleic acid sequence set out in FIG. 1 or FIG. 2, a nucleic acid molecule of the present invention encoding a DR3-V1 or DR3 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule described in FIG. 1 was discovered in a cDNA library derived from cells of a human testis tumor. Also illustrative of the invention, the nucleic acid molecule described in FIG. 2 was discovered in a human HUVEC cDNA library. In addition, the genes of the present invention have also been identified in cDNA libraries of the following tissues: fetal liver, fetal brain, tonsil and leukocyte. Furthermore, multiple forms of DR3 transcript are seen in Northern Blots and PCR reactions indicating that multiple variants of the transcript exists, possibly due to alternate splicing of the message.

The DR3-V1 (formerly called DDCR) gene contains an open reading frame encoding a protein of about 428 amino acid residues whose initiation codon is at position 198-200 of the nucleotide sequence shown in FIG. 1 [SEQ ID NO.1], with a leader sequence of about 35 amino acid residues, and a deduced molecular weight of about 47 kDa. Of known members of the TNF receptor family, the DR3-V1 polypeptide of the invention shares the greatest degree of

homology with human TNF RI. The DR3-V1 polypeptide shown in FIG. 1 [SEQ ID NO:2] is about 20% identical and about 50% similar to human TNF RI.

The DR3 gene contains an open reading frame encoding a protein of about 417 amino acid residues whose initiation codon is at position 1-3 of the nucleotide sequence shown in FIG. 2 [SEQ ID NO:3], with a leader sequence of about 24 amino acid residues, and a deduced molecular weight of about 43 kDa. Of known members of the TNF receptor family, the DR3 polypeptide of the invention shares the greatest degree of homology with human TNF RI. The DR3 polypeptide shown in FIG. 2 [SEQ ID NO:3] is about 20% identical and about 50% similar to human TNF RI.

As indicated, the present invention also provides the mature form(s) of the DR3-V1 and DR3 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature DR3-V1 or DR3 polypeptides having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97456 and 97757, respectively, and as shown in Figure 1 (SEQ ID NO:2) and Figure 2 (SEQ ID NO:4). By the mature DR3-V1 or DR3 protein having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97456 and 97757, respectively, is meant the mature form(s) of the DR3-V1 or DR3 protein produced by expression in a mammalian cell (e.g., COS cells, as

described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature DR3-V1 or DR3 having the amino acid sequence encoded by the cDNA clones contained in ATCC Deposit No. 97456 and 97757, respectively, may or may not differ from the predicted "mature" DR3-V1 protein shown in Figure 1 (amino acids from about 36 to about 428) or DR3 protein shown in Figure 2 (amino acids from about 24 to about 417) depending on the accuracy of the predicted cleavage site based on computer analysis.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) and von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequences of the complete DR3-V1 and DR3 polypeptides of the present invention were analyzed by a computer program ("PSORT"), *see*, K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 35 and 36 in Figure 1 (SEQ ID NO:2) and between amino acids 24 and 25 in Figure 2 (SEQ ID NO:4). Thereafter, the complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heine. von Heinje, *supra*. Thus, the leader sequence for the DR3-V1 protein is predicted to consist of amino acid residues 1- 35 in Figure 1 (SEQ ID NO:2), while the predicted mature DR3-V1 protein consists of residues 36-428. The leader sequence for the DR3 protein is predicted to consist

of amino acid residues 1- 24 in Figure 2 (SEQ ID NO:4), while the predicted mature DR3 protein consists of residues 25-417.

As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, as well as the variability of cleavage sites for leaders in different known proteins, the actual DR3-V1 polypeptide encoded by the deposited cDNA comprises about 428 amino acids, but may be anywhere in the range of 410-440 amino acids; and the actual leader sequence of this protein is about 35 amino acids, but may be anywhere in the range of about 25 to about 45 amino acids. The actual DR3 polypeptide encoded by the deposited cDNA comprises about 417 amino acids, but may be anywhere in the range of 400-430 amino acids; and the actual leader sequence of this protein is about 24 amino acids, but may be anywhere in the range of about 14 to about 34 amino acids.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR3-V1 DNA molecules comprising an open reading frame (ORF) shown in FIG. 1 [SEQ

ID NO:1] and further include DNA molecules which comprise a sequence substantially different than all or part of the ORF whose initiation codon is at position 198-200 of the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] but which, due to the degeneracy of the genetic code, still encode the DR3-V1 polypeptide or a fragment thereof. Isolated nucleic acid molecules of the present invention also include DR3 DNA molecules comprising an open reading frame (ORF) shown in FIG. 2 [SEQ ID NO:3] and further include DNA molecules which comprise a sequence substantially different than all or part of the ORF whose initiation codon is at position 1-3 of the nucleotide sequence shown in FIG. 2 [SEQ ID NO:3] but which, due to the degeneracy of the genetic code, still encode the DR3 polypeptide or a fragment thereof. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR3-V1 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97456 on March 1, 1996. The invention provides isolated nucleic acid molecules encoding the DR3 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97757 on October 10, 1996. Preferably, these nucleic acid molecules will encode the mature polypeptide encoded by the above-described deposited cDNA clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figure 1 (SEQ ID NO:1) or FIG. 2 (SEQ ID NO:3) or the nucleotide sequence of the DR3-V1 or DR3 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated DNA molecules and fragments thereof are useful as DNA probes for gene mapping by *in situ* hybridiz DR3-V1 or DR3 gene in human tissue (including testis tumor tissue) by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By fragments of an isolated DNA molecule having the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3] are intended DNA fragments at least 20 bp, and more preferably at least 30 bp in length, which are useful as DNA probes as discussed above. Of course larger DNA fragments 50-1500 bp in length are also useful as DNA probes according to the present invention, as are DNA fragments corresponding to most, if not all, of the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3]. By a fragment at least 20 bp in length, for example, is intended fragments which include 20 or more bases from the nucleotide sequence in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3].

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising the DR3 extracellular domain (amino acid residues from about 36 to about 212 in FIG. 1 [SEQ ID NO:2]); a polypeptide comprising the DR3 transmembrane domain (amino acid residues from about 213 to about 235 in FIG. 1 [SEQ ID NO:2]); a polypeptide comprising the DR3 intracellular domain (amino acid residues from about 214 to about 428 in FIG. 1 [SEQ ID NO:2]); and a polypeptide comprising the DR3 death domain (amino acid residues from about 353 to about 419 in FIG. 1 [SEQ ID NO:2]). Since the location of these domains have been predicted by computer graphics, one of ordinary skill would appreciate that the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to 15 residues) depending on the criteria used to define the domain.

Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding epitope-bearing portions of the DR3-V1 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a

polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in Figure 1 (SEQ ID NO:2). The inventors have determined that the above polypeptide fragments are antigenic regions of the DR3-V1 protein. Methods for determining other such epitope-bearing portions of the DR3-V1 protein are described in detail below.

Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding epitope-bearing portions of the DR3 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding the corresponding regions to those epitope-bearing regions of the DR3-V1 protein disclosed above. Methods for determining other such epitope-bearing portions of the DR3 protein are described in detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit 97456 or ATCC Deposit 97757. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about

15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

5 By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1) or Figure 2 (SEQ ID NO:3)).

10 Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the DR3-V1 cDNA shown in Figure 1 (SEQ ID NO:1)), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof
15 (e.g., practically any double-stranded cDNA clone).

20 As indicated, nucleic acid molecules of the present invention which encode the DR3-V1 or DR3 polypeptide may include, but are not limited to the coding sequence for the mature polypeptide, by itself, the coding sequence for the mature polypeptide and additional sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome
25 binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain

preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37:767 (1984), for instance.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode for fragments, analogs or derivatives of the DR3-V1 or DR3 polypeptide. Variants may occur naturally, such as an allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions, or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions, or additions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the full-length DR3-V1 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence; (b) nucleotide sequence encoding the full-length DR3 polypeptide having the complete amino acid sequence in Figure 2 (SEQ ID NO:4), including the predicted leader sequence; (c) a nucleotide sequence encoding the mature DR3-V1 polypeptide (full-length polypeptide with the leader removed) having the amino acid sequence at positions

about 36 to about 428 in Figure 1 (SEQ ID NO:2); (d) a nucleotide sequence encoding the full-length DR3-V1 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97456; (e) a nucleotide sequence encoding the full-length DR3 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97757; (f) a nucleotide sequence encoding the mature DR3-V1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456; (g) a nucleotide sequence encoding the mature DR3-V1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97757; (h) a nucleotide sequence that encodes the DR3 extracellular domain, (i) a nucleotide sequence that encodes the DR3 transmembrane domain, (j) a nucleotide sequence that encodes the DR3 intracellular domain, and (k) a nucleotide sequence that encodes the DR3 death domain; or (l) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k) above.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a DR3-V1 or DR3 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the DR3-V1 or DR3 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually

among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1, Figure 2 or to the nucleotide sequences of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1), Figure 2 (SEQ ID NO:3) or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having DR3 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having DR3 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DDCR activity include, *inter alia*, (1) isolating the DR3-V1 or DR3 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal

location of the DR3-V1 or DR3 gene, as described in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DR3-V1 or DR3 mRNA expression in specific tissues.

5 Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1), Figure 2 (SEQ ID NO:3) or to the nucleic acid sequence of the deposited cDNAs which do, in fact, encode a polypeptide having DR3 protein activity. By "a polypeptide having DR3 activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DR3 protein of the invention (either the full-length protein or, preferably, the mature protein), as measured in a particular biological assay. For example, DR3 protein activity can be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)), and as set forth in Example 7, below. In MCF7 cells, plasmids encoding full-length DR3 or a candidate death domain containing receptors are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR3 will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin *et al.*, *Cell* 85, 803-815 (1996); M. Tewari *et al.*, *J Biol Chem* 270, 3255-60 (1995)), DR3-induced apoptosis is blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. In addition, apoptosis induced by DR3 is also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S).

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99%

identical to the nucleic acid sequence of the deposited cDNA or the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or Figure 2 (SEQ ID NO:3) will encode a polypeptide "having DR3 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DR3 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Polynucleotide assays

This invention is also related to the use of the DR3-V1 or DR3 polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DR3-V1 or DR3 associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DR3-V1 or DR3 or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DR3-V1 or DR3 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue

biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al.*, *Nature* 324:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding DR3-V1 or DR3 can be used to identify and analyze DR3-V1 or DR3 expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DR3-V1 or DR3 RNA or alternatively, radiolabeled DR3-V1 or DR3 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Chromosome assays

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a DR3-V1 or a DR3 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA the is used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man*, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Vectors and Host Cells

The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector, or supplied by the vector itself upon introduction into the host.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-

known promoters. In general, expression constructs will contain sites for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, and Bowes melanoma cells; and plant cells. Hosts for a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods in Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final

preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5- has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

The DR3 and DR3-V1 polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

DR3-V1 or DR3 polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DR3. Among these are applications in treatment of tumors, resistance to parasites, bacteria and viruses, to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells, to treat restenosis, graft vs. host disease, to regulate anti-viral responses and to prevent certain autoimmune diseases after stimulation of DR3 by an agonist. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DR3 Polypeptides and Fragments

The invention further provides an isolated DR3-V1 or DR3 polypeptide having the amino acid sequence shown in FIG. 1 [SEQ ID NO:2] and FIG. 2[SEQ ID NO:4], respectively, or a fragment thereof. It will be recognized in the art that some amino acid sequence of DR3-V1 or DR3 can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which

make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DR3-V1 or DR3 protein which show substantial DR3 protein activity or which include regions of DR3-V1 or DR3 such as the protein fragments discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in J.U. Bowie *et al.*, *Science* 247:1306-1310 (1990).

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DR3-V1 or DR3 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al.* *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- α to only one of the two known types of TNF receptors. Thus, the DR3-V1 or DR3 receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Amino acids in the DR3-V1 or DR3 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the DR3-V1 or DR3 polypeptide is substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988).

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA including the leader, the mature polypeptide encoded by the deposited the cDNA minus the leader (i.e., the mature protein), the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4) including the leader, the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4) minus the leader, the extracellular domain, the transmembrane domain, the intracellular domain, soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain as well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA clones, to the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4), and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DR3-V1 or DR3 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DR3-V1 or DR3 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2), or Figure 2 (SEQ ID NO:4) or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The present inventors have discovered that the DR3-V1 polypeptide is a 428 residue protein exhibiting three main structural domains. First, the ligand binding domain was identified within residues from about 36 to about 212 in FIG. 1 [SEQ ID NO:2]. Second, the transmembrane domain was identified within residues from about 213 to about 235 in FIG. 1 [SEQ ID NO:2]. Third, the intracellular domain was identified within residues from about 236 to about 428 in FIG. 1 [SEQ ID NO:2]. Importantly, the intracellular domain includes a death domain at residues from about 353 to about 419. Further preferred fragments of the polypeptide shown in FIG. 1 [SEQ ID NO:2] include the mature protein from residues about 36 to about 428 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain.

The present inventors have also discovered that the DR3 polypeptide is a 417 residue protein exhibiting three main structural domains. First, the ligand binding domain was identified within residues from about 25 to about 201 in FIG. 2 [SEQ ID NO:4]. Second, the transmembrane domain was identified within residues from about 202 to about 224 in FIG. 2 [SEQ ID NO:4]. Third, the

intracellular domain was identified within residues from about 225 to about 417 in FIG. 2 [SEQ ID NO:4]. Importantly, the intracellular domain includes a death domain at residues from about 342 to about 408. Further preferred fragments of the polypeptide shown in FIG. 2 [SEQ ID NO:4] include the mature protein from residues about 25 to about 417 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain. As one of skill in the art will recognize, the full length polypeptides encoded by the DR3-V1 and DR3 cDNA differ only in the amino acid sequence of the leader peptide. The first 24 amino acids of the polypeptide shown in Figure 1 are replaced by the first 13 amino acids shown in Figure 2 but the rest of the amino acid sequence is the same. Thus, both the DR3-V1 cDNA and DR3 cDNA encode an identical mature protein having the same biological activity.

Thus, the invention further provides DR3-V1 or DR3 polypeptides encoded by the deposited cDNA clones including the leader and DR3-V1 or DR3 polypeptide fragments selected from the mature protein, the extracellular domain, the transmembrane domain, the intracellular domain, and the death domain.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part

of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, J.G. Sutcliffe *et al.*, "Antibodies that react with predetermined sites on proteins," *Science* 219:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* 37:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR3-specific antibodies include: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in Figure 1 (SEQ ID NO:2). In addition, antigenic polypeptides or peptides include polypeptides

comprising the amino acid residues that are the corresponding residues to those polypeptides of DR3-V1 disclosed above. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR3-V1 and DR3 protein.

5 The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. R.A. Houghten, "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA* 82:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

10 As one of skill in the art will appreciate, DR3-V1 or DR3 polypeptides of the present invention, and the epitope-bearing fragments thereof, described above, can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al.*, *Nature* 331:84- 86 (1988)).

15 Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR3-V1 or DR3 protein or protein fragment alone (Fountoulakis *et al.*, *J Biochem* 270:3958-3964 (1995)).

20

Polypeptide assays

25 The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR3-V1 or DR3 protein, or the soluble form thereof, in cells and tissues, including determination of normal and

abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR3-V1 or DR3, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as an DR3 protein of the present invention, or a soluble form thereof, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Assaying DR3-V1 or DR3 protein levels in a biological sample can occur using any art-known method. Preferred for assaying DR3-V1 or DR3 protein levels in a biological sample are antibody-based techniques. For example, DR3-V1 or DR3 protein expression in tissues can be studied with classical immunohistological methods. M. Jalkanen *et al.*, *J. Cell. Biol.* 101:976-985 (1985); M. Jalkanen *et al.*, *J. Cell. Biol.* 105:3087-3096 (1987).

Other antibody-based methods useful for detecting DR3-V1 or DR3 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{125}I , ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (D.V. Goeddel *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-

609 (1986), Cold Spring Harbor; B. Beutler and A. Cerami, *Annu. Rev. Biochem.* 57:505-518 (1988); L.J. Old, *Sci. Am.* 258:59-75 (1988); W. Fiers, *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DR3-V1 or DR3 of the present invention. Cells which express the DR3-V1 or DR3 polypeptide and are believed to have a potent cellular response to DR3-V1 or DR3 ligands include lymphocytes, fibroblasts, macrophages, synovial cells, activated T-cells, lymphoblasts and epithelial cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (J.C. Ameisen, *AXDS* 8:1197-1213 (1994); P.H. Krammer *et al.*, *Curr. Opin. Immunol.* 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus, immune-related glomerulonephritis, and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft versus host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion

injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia, and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3-V1 or DR3 polypeptide an effective amount of DR3-V1 or DR3 ligand, analog or an agonist capable of increasing DR3-V1 or DR3 mediated signaling. Preferably, DR3-V1 or DR3 mediated signaling is increased to treat a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist can include soluble forms of DR3-V1 or DR3 and monoclonal antibodies directed against the DR3-V1 or DR3 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the, DR3-V1 or DR3 polypeptide an effective amount of an antagonist capable of decreasing DR3-V1 or DR3 mediated signaling. Preferably, DR3-V1 or DR3 mediated signaling is decreased to treat a disease wherein increased apoptosis or NFkB expression is exhibited. An antagonist can include soluble forms of DR3-V1 or DR3 and monoclonal antibodies directed against the DR3-V1 or DR3 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such

an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science* 246:181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA

encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in L.A. Tartaglia and D.V. Goeddel, *J. Biol. Chem.* 267:4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR3-V1 or DR3 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DR3-V1 or DR3 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments,

transforming growth factor β , neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and β -amyloid peptide (*Science* 267:1457-1458 (1995)). Further preferred agonists include polyclonal and monoclonal antibodies raised against the DR3-V1 or DR3 polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in L.A. Tartaglia *et al.*, *Proc. Natl. Acad. Sci. USA* 88:9292-9296 (1991); and L.A. Tartaglia and D.V. Goeddel, *supra*. See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *E1B*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus *yl 34.5*), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and α -Hexachlorocyclohexane).

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense

RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the receptor.

Further antagonist according to the present invention include soluble forms of DR3-V1 or DR3, i.e., DR3-V1 or DR3 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR3-V1 or DR3 mediated signaling by competing with the cell surface DR3-V1 or DR3 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, e.g., IgGFC-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (D.P. Hughes and I.N. Crispe, *J. Exp. Med.* 182:1395-1401 (1995)).

The experiments set forth in Examples 6 and 7 demonstrate that DR3 is a death domain-containing molecule capable of triggering both apoptosis and NF-kB activation, two pathways dominant in the regulation of the immune system. The experiments also demonstrate the internal signal transduction machinery of this novel cell death receptor. In addition, the experiments set forth below demonstrate that DR3-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. Importantly, apoptosis induced by DR3 was

also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S), which were previously shown to inhibit death signaling by Fas/APO-1 and TNFR-1. Thus, inhibitors of ICE-like proteases, FADD-DN and FLICE-DN/MACHa1C360S could also be used as antagonists for DR3 activity.

The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')₂ fragments) which are capable of binding an antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods using DR3-V1 or DR3 immunogens of the present invention. As indicated, such DR3-V1 or DR3 immunogens include the full length DR3-V1 or DR3 polypeptide (which may or may not include the leader sequence) and DR3-V1 or DR3 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Proteins and other compounds which bind the DR3-V1 or DR3 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (J. Gyuris *et al.*, *Cell* 75:791-803 (1993); A.S. Zervos *et al.*, *Cell* 72:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture compounds which bind to either the DR3-V1 or DR3 ligand binding domain or to the DR3-V1 or DR3 intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor

family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, the DR3-V1 or DR3 ligand, TNF- α , lymphdotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40, CD27, CD30, 4-1BB, OX40 and nerve growth factor (NGF).

Representative therapeutic applications of the present invention are discussed in more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4⁺ T-lymphocytes. Recent reports estimate the daily loss of CD4⁺ T cells to be between 3.5×10^7 and 2×10^9 cells (X. Wei *et al.*, *Nature* 373:117-122 (1995)). One cause of CD4⁺ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (J.C. Ameisen, *AIDS* 8:1197-1213 (1994); T.H. Finkel and N.K. Banda, *Curr. Opin. Immunol.* 6:605-615(1995); C.A. Muro-Cacho *et al.*, *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4⁺ T-lymphocyte depletion are tightly correlated in different animal models of AIDS (T. Brunner *et al.*, *Nature* 373:441-444 (1995); M.L. Gougeon *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)), and apoptosis is not observed in those animal models in which viral replication does not result in AIDS. *Id.* Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (A.D. Badley *et al.*, *J. Virol.* 70:199-206 (1996)). Further, the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes. *Id.* Thus, by the invention, a method for treating HIV⁺ individuals is provided which involves administering an antagonist

of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonist of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR3-V1 or DR3 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel-Disease.

DR3, like TNFR1, also activates the NF-kB transcription factor, which is very closely associated with the stimulation of cytokine (e.g., IL-8) and adhesion molecule (e.g., ELAM) transcription. Hence, like TNF, the ligand (or agonist) for DR3 and DR3-V1 may in some circumstances be proinflammatory, and antagonists may be useful reagents for blocking this response. Thus, DR3 and DR3-V1 antagonists may be useful for treating inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR3, soluble DR3, agonist or antagonist mABs may be used to treat this form of cancer. Further, soluble

DR3 or neutralizing mABs may be used to treat various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

Modes of Administration

5 The agonist or antagonists described herein can be administered *in vitro*,
ex vivo, or *in vivo* to cells which express the receptor of the present invention. By
administration of an "effective amount" of an agonist or antagonist is intended an
amount of the compound that is sufficient to enhance or inhibit a cellular response
to a TNF-family ligand and include polypeptides. In particular, by administration
10 of an "effective amount" of an agonist or antagonists is intended an amount
effective to enhance or inhibit DR3-V1 or DR3 mediated apoptosis. Of course,
where apoptosis is to be enhanced, an agonist according to the present invention
can be co-administered with a TNF-family ligand. One of ordinary skill will
appreciate that effective amounts of an agonist or antagonist can be determined
15 empirically and may be employed in pure form or in pharmaceutically acceptable
salt, ester or prodrug form. The agonist or antagonist may be administered in
compositions in combination with one or more pharmaceutically acceptable
excipients.

20 It will be understood that, when administered to a human patient, the total
daily usage of the compounds and compositions of the present invention will be
decided by the attending physician within the scope of sound medical judgement.
The specific therapeutically effective dose level for any particular patient will
depend upon factors well known in the medical arts.

25 As a general proposition, the total pharmaceutically effective amount of
DDCR polypeptide administered parenterally per dose will be in the range of
about 1 µg/kg/day to 10 mg/kg/day of patient body weight, although, as noted
above, this will be subject to therapeutic discretion. More preferably, this dose is

at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the DDCR agonists or antagonists is typically administered at a dose rate of about 1 µg/kg/hour to about 50 µg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

Pharmaceutical compositions are provided comprising an agonist or antagonist and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand, clinical side effects can be reduced by using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DR3-V1 or DR3 polypeptides, DR3-V1 or DR3 polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

5

Example 1

Expression and Purification in E. coli

10

The DNA sequence encoding the mature DR3-V1 protein in the deposited cDNA clone (ATCC No. 97456) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DR3-V1 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

15

The following primers are used for expression of DR3 extracellular domain in *E. coli* 5' primer 5'-GCGCCATGGGGGCCCCGGCGGCAG-3' (SEQ ID NO:7) contains an NcoI site and 15 nucleotide starting from 290 nucleotide to 304 FIG. 1. 3' primer 5'-GCGAAGCTTCTAGGACCCAGAACATCTGCC-3' (SEQ ID NO:8) contains a *Hind*III site, a stop codon and 18 nucleotides complimentary to nucleotide from 822 to 840 in FIG. 1. Vector is pQE60. The protein is not tagged.

20

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in these examples. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS").

25

The amplified DR3-V1 DNA and the vector pQE60 both are digested with *Nco* I and *Hind*III and the digested DNAs are then ligated together. Insertion of the DDCR protein DNA into the restricted pQE60 vector places the DR3-V1

protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of DR3-V1 protein.

5 The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative
10 example described herein. This strain, which is only one of many that are suitable for expressing DR3-V1 protein, is available commercially from Qiagen.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis.

15 Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

20 The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD600") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lacI* repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified
25 from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography

to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 μ /ml.

Example 2

Expression in Mammalian Cells

5 Most of the vectors used for the transient expression of a given gene sequence in mammalian cells carry the SV40 origin of replication. This allows the replication of the vector to high copy numbers in cells (e.g., COS cells) which express the T antigen required for the initiation of viral DNA synthesis. Any other mammalian cell line can also be utilized for this purpose.

10 A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA
15 splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI, and the early promoter of the cytomegalovirus (CMV). However, also cellular signals can be used (e.g., human actin, promoter). Suitable expression vectors for use in practicing the present invention include, for example,
20 vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human Hela, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7, and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells, and Chinese hamster ovary (CHO)
25 cells.

Alternatively, a gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

5 The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) is a useful marker to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Using this marker, the mammalian cells are grown in increasing amounts of methotrexate for selection and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

10 The expression vectors pCl and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology* 438:44701 (March 1985)), plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites *Bam*HI, *Xba*I and *Asp*718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

20 *Example 2A*

Expression of extracellular soluble domain of DR3-V1 and DR3 in COS cells

25 The expression plasmid, pDR3-V1 HA, is made by cloning a cDNA encoding DR3-V1 (ATCC No. 97456) into the expression vector pcDNAI/Amp (which can be obtained from Invitrogen, Inc.). Expression plasmid, pDR3 HA, is made by cloning a cDNA encoding DR3 (ATCC No. 97757) into the expression vector pcDNAI/Amp.

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cell; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron, and a polyadenylation signal arranged so that a cDNA conveniently can be placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker.

A DNA fragment encoding the entire DR3-V1 or Dr3 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al.*, *Cell* 37:767 (1984). The fusion of the HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is as follows:

The DR3-V1 or DR3 cDNA of the deposit clone is amplified using primers that contained convenient restriction sites, much as described above regarding the construction of expression vectors for expression of DR3-V1 or DR3 in *E. coli* and *S. fugiperda*.

To facilitate detection, purification and characterization of the expressed DR3-V1 or DR3, one of the primers contains a hemagglutinin tag ("HA tag") as described above.

Suitable primers for DR3-V1 include the following, which are used in this example, the 5' primer,

5' CGCGGATCCGCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:9) contains the underlined *Bam*HI site, an ATG start codon and 5 codons thereafter.

Suitable primers for DR3 include the following, which are used in this example, the 5' primer,

5' CGCGGATCCGCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:10)
contains the underlined *Bam*HI site, an ATG start codon and 5 codons thereafter.

The 3' primer for both DR3 and DR3-V1, containing the underlined *Xba*I site, stop codon, hemagglutinin tag and last 14 nucleotide of 3' coding sequence (at the 3' end) has the following sequence:

5'GCGTCTAGATCAAAGCGTAGTCTGGGACGTCGTATGGGTACGGGC
CGCGCTGCA 3' (SEQ ID NO:11).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with *Bam*HI and *Xba*I and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis and gel sizing for the presence of the DR3-V1 or DR3-encoding fragment.

For expression of recombinant DR3-V1 or DR3, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, NY (1989).

Cells are incubated under conditions for expression of DR3-V1 or DR3 by the vector.

Expression of the DR3-V1 HA fusion protein or the DR3 HA fusion protein is detected by radiolabelling and immunoprecipitation, using methods described in, for example Harlow *et al.*, *Antibodies: a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ³⁵S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and then lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as

described by Wilson *et al.* cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE gels and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 2B

Expression and purification of human DR3-V1 and DR3 using the CHO Expression System

The vector pC1 is used for the expression of DR3-V1 or DR3 (ATCC No. 97456 or ATCC No. 97757, respectively) protein. Plasmid pC1 is a derivative of the plasmid pSV2-dhfr [ATCC Accession No. 37146]. Both plasmids contain the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., F.W. Alt *et al.*, *J Biol. Chem.* 253:1357-1370 (1978); J.L. Hamlin and C. Ma, *Biochem. et Biophys. Acta*, 1097:107-143 (1990); M.J. Page and M.A. Sydenham, *Biotechnology* 9:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1,000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

Plasmid pC1 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology* 5:438-447 (March 1985)), plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al.*, *Cell* 41:521-530 (1985)). Downstream from the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: *Bam*HI followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human β -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC1 is digested with the restriction enzyme *Bam*HI and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding DR3-V1 or DR3 in the deposited cDNA clones are amplified using PCR oligonucleotide primers specific to the amino acid carboxyl terminal sequence of the DR3-V1 or DR3 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The 5' oligonucleotide primer for DR3-V1 has the sequence 5' CGCGGATCCGCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:12) containing the underlined *Bam*HI restriction site, which encodes a start AUG, followed by the Kozak sequence and 18 nucleotides of the DR3-V1 coding sequence set out in FIG. 1 beginning with the 1st base of the ATG codon.

The 5' oligonucleotide primer for DR3 has the sequence 5' CGCGGATCCGCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:13) containing the underlined BamHI restriction site, which encodes a start AUG, followed by the Kozak sequence and 18 nucleotides of the DR3 coding sequence set out in FIG. 2 beginning with the first base of the ATG codon.

The 3' primer for both DR3 and DR3-V1 has the sequence 5' CGCGGATCCTCACGGGCCGCGCTGCA 3' (SEQ ID NO:14) containing the underlined *Bam*HI restriction site followed by 17 nucleotides complementary to the last 14 nucleotides of the DR3-V1 or DR3 coding sequence set out in FIG. 1 or FIG. 2, respectively, plus the stop codon.

The restriction sites are convenient to restriction enzyme sites in the CHO expression vectors pC1.

The amplified DR3 or DR3-V1 DNA and the vector pC1 both are digested with *Bam*HI and the digested DNAs then ligated together. Insertion of the DR3-V1 or DR3 DNA into the *Bam*HI restricted vector placed the DR3-V1 or DR3 coding region downstream of and operably linked to the vector's promoter. The sequence of the inserted gene is confirmed by DNA sequencing.

Transfection of CHO-DHFR-cells

Chinese hamster ovary cells lacking an active DHFR enzyme are used for transfection. 5 µg of the expression plasmid C1 are cotransfected with 0.5 µg of the plasmid pSVneo using the lipofecting method (Felgner *et al.*, *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the gene neo from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) and cultivated from 10-14 days. After this period, single clones are trypsinized and then seeded in 6-well petri dishes using different

concentrations of methotrexate (25 nM, 50 nM, 100 nM, 200 nM, 400 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (500 nM, 1 µM, 2 µM, 5 µM). The same procedure is repeated until clones grow at a concentration of 100 µM.

The expression of the desired gene product is analyzed by Western blot analysis and SDS-PAGE.

Example 3

Cloning and expression of the soluble extracellular domain of DR3-V1 and DR3 in a baculovirus expression system

The cDNA sequence encoding the soluble extracellular domain of DR3-V1 or DR3 protein in the deposited clone (ATCC No. 97456 or ATCC No. 97757, respectively) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DR3-V1 has the sequence 5' CGCGGATCC GCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:15) containing the underlined *Bam*HI restriction enzyme site followed by a Kozak sequence and a number of bases of the sequence of DR3-V1 of FIG. 1. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DR3-V1 provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by M. Kozak, *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 5' primer for DR3 has the sequence 5' CGCGGATCC GCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:16) containing the underlined *Bam*HI restriction enzyme site followed by a Kozak sequence and a number of bases of the sequence of DR3 of FIG. 2. Inserted into an expression

vector, as described below, the 5' end of the amplified fragment encoding DR3 provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by M. Kozak, *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

5 The 3' primer for both DR3 and DR3-V1 has the sequence 5' GCGAGATCTAGTCTGGACCC AGAACATCTGCCTCC 3' (SEQ ID NO:17) containing the underlined XbaI restriction followed by nucleotides complementary to the DR3-V1 or DR3 nucleotide sequence set out in FIG. 1 or FIG.2, respectively, followed by the stop codon.

10 The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with *Bam*HI and *Asp*718 and again is purified on a 1% agarose gel. This fragment is designated herein F2.

15 The vector pA2 is used to express the DR3-V1 or DR3 protein in the baculovirus expression system, using standard methods, such as those described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the Autograph californica nuclear polyhedrosis virus (ACMNPV) followed by
20 convenient restriction sites. For an easy selection of recombinant virus the beta-galactosidase gene from *E. coli* is inserted in the same orientation as the polyhedron promoter and is followed by the polyadenylation signal of the polyhedron gene. The polyhedron sequences are flanked at both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA
25 to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription, translation, trafficking and the like, such as an in-frame AUG and a signal peptide,

as required. Such vectors are described in Luckow *et al.*, *Virology* 170:31-39 (1989), among others.

The plasmid is digested with the restriction enzymes *Bam*HI and *Xba*I and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V2".

Fragment F2 and the dephosphorylated plasmid V2 are ligated together with T4 DNA ligase. *E. coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human DDCR gene by digesting DNA from individual colonies using *Bam*HI and *Xba*I and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DR3-V1 or pBac DR3.

5 µg of the plasmid pBac DR3-V1 or pBac DR3 is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac DR3-V1 are mixed in a sterile well of a microliter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. A clone containing properly inserted DR3-V1 or DR3 is identified by DNA analysis including restriction mapping and sequencing. This is designated herein as V- DR3-V1 or V-DR3.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V- DR3-V1 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg). 42 hours later, 5 gCi of ³⁵S-methionine and 5 µCi ³⁵S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

Example 4

A. Tissue distribution of DR3-V1 gene expression

Northern blot analysis is carried out to examine DR3-V1 gene (ATCC No. 97456) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR3-V1 protein (SEQ ID NO:1) is labeled with ³²P using the *rediprime*TM DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100TM column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DR3-V1 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using ExpressHybTM hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures. Expression of DR3-V1 was detected in tissues enriched in lymphocytes including peripheral blood leukocytes (PBLs), thymus, spleen, colon, and small intestine. DR3-V1 expression appears to be restricted to lymphocyte compartments, it can be envisaged that DR3-V1 plays a role in lymphocyte homeostasis.

B. Tissue distribution of DR3 gene expression

Northern blot analysis is carried out to examine DR3 gene (ATCC No. 97757) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide

sequence of the DR3 protein (SEQ ID NO:1) is labeled with ^{32}P using the rediprime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DR3 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures.

Expression of DR3 was detected in tissues enriched in lymphocytes including peripheral blood leukocytes (PBLs), thymus, spleen, colon, and small intestine. By contrast, TNFR-1 is ubiquitously expressed and Fas/APO-1 is expressed in lymphocytes, liver, heart, lung, kidney, and ovary (Watanabe-Fukunaga *et al.*, *J. Immunol* 148:1274-9 (1992)).

DR3 expression appears to be restricted to lymphocyte compartments, it can be envisaged that DR3 plays a role in lymphocyte homeostasis.

C. Northern Blot analysis of DR3 in various cell lines

Methods

Cells

Unless stated otherwise, cell lines were obtained from the American Type Culture Collection (Rockville, MD). The myeloid (Koeffler *et al.* (1980); Koeffler (1983); Harris and Ralph (1985); and Tucker *et al.* (1987)) and B-cell lines (Jonak *et al.* (1922)) studied represent cell types at different stages of the differentiation pathway. KG1a and PLB 985 cells (Tucker *et al.* (1987)) were obtained from H.P. Koeffler (UCLA School of Medicine). BJA-B was from Z. Jonak

(SmithKline Beecham). TF274, a stromal cell line exhibiting osteoblastic features, was generated from the bone marrow of a healthy male donor (Z. Jonak and K.B. Tan, unpublished). Primary carotid artery endothelial cells were purchased from Clonetics Corp. (San Diego, CA) and monocytes were prepared by differential centrifugation of peripheral blood mononuclear cells and adhesion to tissue culture dish. CD19+, CD4+ and CD8+ cells (>90% pure) were isolated with cell type specific immunomagnetic beads (Drynal, Lake Success, NY).

RNA Analysis

Total RNA of adult tissues were purchased from Clontech (Palo Alto, CA). Total RNA was extracted from cell lines (in exponential growth phase) and primary cells with TriReagent (Molecular Research Center, Inc., Cincinnati, OH). 5 to 7.5 µg of total RNA was fractionated in a 1% agarose gel containing formaldehyde cast in a Wide Mini-Sub Cell gel tray (Bio-Rad, Hercules, CA) as described (Sambrook, et al.) with slight modifications. The formaldehyde concentration was reduced to 0.5M and the RNA was stained prior to electrophoresis with 100 µg/ml of etidium bromide that was added to the loading buffer. After electrophoresis with continuous buffer recirculation (60 volts/90 min), the gel was photographed and the RNA was transferred quantitatively to Zeta-probe nylon membrane (Biorad, Hercules, CA) by vacuum-blotting with 25 mM NaOH for 90 min. After neutralization for 5-10 min, with 1M Tris-HCl, pH 7.5 containing 3M NaCl, the blots were prehybridized with 50% formamide, 8% dextran sulfate, 6xSSPE, 0.1% SDS and 100 µg/ml of sheared and denatured salmon sperm DNA for at least 30 min at 42°C. cDNA inserts labeled with ³²P-dCTP by random priming (Stratagene, La Jolla, CA), were denatured with 0.25M NaOH (10 min at 37°C) and added to the prehybridization solution. After 24-65 hr at 42°C, the blots were washed under high stringency conditions (Sambrook, et al.) and exposed to X-ray films.

Results

Expression of DR3 was assessed by Northern blot in the following cell lines: TF274 (bone marrow stromal); MG63, TE85 (osteosarcoma); K562 (erythroid); KG1a, KG1, PLB985, HL60, U937, TNHP-1 (myeloid); REH, BJAB, Raji, IM-9 (B cell); Sup-T1, Jurkat, H9, Molt-3 (T cell); RL95-2 (endometrial carcinoma); MCF-7 (breast cancer); BE, HT29 (colon cancer); IMR32 (neuroblastoma) and could only be detected in KG1a cells. DR3 expression was detected in several lymphoblast cell lines. In the purified human hematopoietic cell populations, DR3 was weakly expressed in CD19+ cells, and more highly expressed in monocytes. However the highest levels were observed in T cells (CD4+ or CD8+) upon stimulation with PMA and PHA, indicating that DR3 probably plays a role in the regulation of T cell activation.

Example 5

Intracellular Signaling Molecules used by DR3 Protein

In vitro and *in vivo* binding studies were undertaken to investigate DR3 signaling pathways. Since DR3 contains a death domain, the inventors postulated that DR3, like TNFR-1 and Fas/APO-1, may transduce signals by recruiting death domain-containing adapter molecules (DAMs) such as FADD, TRADD, and RIP.

Experimental Design

In vitro binding experiments were performed as described previously (A.M. Chinnaiyan *et al.*, *Cell* 81: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem* 270: 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO* 14: 5579-5588 (1995)). Briefly, the cytoplasmic domains of DR3 (amino acid residues 215-393 (Figure 2)) and the death domain mutant ΔDR3 (amino acid residues 215-321 (Figure 2)) were amplified by PCR using appropriate templates and primers into pGSTag. pGSTag

and pGSTag-TNFR-1 were described previously (A.M. Chinnaiyan *et al.*, *Cell* 81: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem* 270: 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO* 14: 5579-5588 (1995)). GST and GST fusion proteins were prepared from *E.coli* strain BL21(DE3)pLysS using standard published procedures and the recombinant proteins immobilized onto glutathione-agarose beads. ³⁵S-Labeled FADD, RIP and TRADD were prepared by in vitro transcription-translation using the TNT or T7 or SP6-coupled reticulocyte lysate system from Promega according to manufacturer's instructions, using pcDNA3 AU1-FADD (A.M. Chinnaiyan *et al.*, *Cell* 81: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem* 270: 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO* 14: 5579-5588 (1995)), pRK myc-TRADD (H. Hsu *et al.*, *Cell* 81: 495-504 (1995)), or pRK myc-RIP (H. Hsu *et al.*, *Immunity* 4: 387-396 (1996)) as template. Following translation, equal amounts of total ³⁵S-labeled reticulocyte lysate were diluted into 150 µl GST binding buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 1% NP-40) and incubated for 2 hrs. at 4°C with the various GST fusion proteins complexed to beads, following the beads were pelleted by plus centrifugation, washed three times in GST buffer, boiled in SDS-sample buffer and resolved on a 12.5% SDS-PAGE. Bound proteins were visualized following autoradiography at -80°C. *In vitro* translated ³⁵S-labeled RIP, TRADD and FADD were incubated with glutathione beads containing GST alone or GST fusions of the cytoplasmic domain of Fas, TNFR-1, DR3 (215-393), or DDR3 (215-321). After the beads were washed, retained proteins were analyzed by SDS-PAGE and autoradiography. The gel was Coomassie stained to monitor equivalency of loading.

To demonstrate the association of DR3 and TRADD in vivo, constructs encoding Flag-TNFR-1 and Flag-ΔTNFR-1 were used. The Flag-TNFR-1 and Flag-ΔTNFR-1 constructs were described elsewhere (A.M. Chinnaiyan *et al.*, *J Biol Chem* 271: 4961-4965 (1996)). The constructs encoding Flag-TNFR-1 and Flag-ΔTNFR-1 were described elsewhere (A.M. Chinnaiyan *et al.*, *J Biol Chem*

271: 4961-4965 (1996)). To facilitate epitope tagging, DR3 and Δ DR3 (1-321) were cloned into the IBI Kodak FLAG plasmid (pCMV1FLAG) utilizing the signal peptide provided by the vector. 293 cells (2×10^6 /100mm plate) were grown in DMEM media containing 10% heat-inactivated fetal bovine serum containing penicillin G, streptomycin, glutamine, and non-essential amino acids. Cells were transfected using calcium phosphate precipitation with the constructs encoding the indicated proteins in combination with pcDNA3-CrmA (M. Tewari *et al.*, *J Biol Chem* 270: 3255-60 (1995)) to prevent cell death and thus maintain protein expression. Cells were lysed in 1 ml lysis buffer (50mM Hepes, 150mM NaCl, 1mM EDTA, 1% NP-40, and a protease inhibitor cocktail). Lysates were immunoprecipitated with a control monoclonal antibody or anti-Flag antibody for at least 4 hrs, at 4°C as previously described (A.M. Chinnaiyan *et al.*, *J Biol Chem* 271: 4961-4965 (1996)). The beads were washed with lysis buffer 3X, but in the case of TRADD binding, the NaCl concentration was adjusted to 1M. The precipitates were fractioned on 12.5% SDS-PAGE and transferred to nitrocellulose. Subsequent Western blotting was performed as described elsewhere (H. Hsu *et al.*, *Cell* 84: 299-308 (1996); A.M. Chinnaiyan *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). After 24-32 hours, extracts were prepared and immunoprecipitated with a control monoclonal antibody or anti-Flag monoclonal antibody (IBI Kodak). Western analysis indicated that myc-TRADD and death receptor expression levels were similar in all samples. Coprecipitating myc-TRADD was detected by immunoblotting using an anti-myc HRP conjugated antibody (Boehringer Mannheim).

Results

As an initial screen, *in vitro* translated radiolabeled DAMs were precipitated with various glutathione S-transferase (GST) fusion proteins immobilized on glutathione-Sepharose beads. As predicted from previous studies (A.M. Chinnaiyan *et al.*, *Cell* 81: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem*

270: 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO 14*: 5579-5588 (1995); H. Hsu
et al., *Cell 81*: 495-504 (1995)), FADD associated with the GST-Fas cytoplasmic
domain while TRADD associated with the GST-TNFR-1 cytoplasmic domain. In
addition, there was a direct, albeit weak, interaction between RIP and
5 GST-TNFR-1. Interestingly, GST-DDCR associated specifically with TRADD,
but not FADD or RIP. Furthermore, a truncated death domain mutant of DR3
(GST-DDR3) failed to interact with TRADD. To demonstrate the association of
DR3 and TRADD *in vivo*, 293 cells were transiently transfected with plasmids
that direct the synthesis of myc-epitope tagged TRADD (myc-TRADD) and
10 Flag-epitope tagged DR3 (Flag-DR3), Flag-TNFR-1 or mutants. Consistent with
the *in vitro* binding study, TRADD specifically coprecipitated with DR3 and
TNFR-1, but not with the death domain mutants, DDR3 and DTNFR-1. Thus,
it appears that DR3, like TNFR-1, may activate downstream signaling cascades
by virtue of its ability to recruit the adapter molecule TRADD.

15 Overexpression of TRADD induces apoptosis and NF-kB activation-two
of the most important activities signaled by TNFR-1 (H. Hsu *et al.*, *supra*). Upon
oligomerization of TNFR-1 by trimeric TNF, TRADD is recruited to the receptor
signaling complex (H. Hsu *et al.*, *Cell 84*:299-308 (1996)). TRADD can then
recruit the following signal transducing molecules: 1) TRAF2, a TNFR-2- and
20 CD40 - associated molecule (M. Rothe *et al.*, *Cell 78*: 681-92 (1994); M. Rothe
et al., *Science 269*:1424-1427 (1995)), that mediates NF-kB activation, 2) RIP,
originally identified as a Fas/APO-1-interacting protein by two-hybrid analysis
(B.Z. Stanger *et al.*, *Cell 81*: 513-23 (1995)), that mediates NF-kB activation and
apoptosis (H. Hsu *et al.*, *Immunity 4*: 387-396 (1996)), and 3) FADD, a
25 Fas/APO-1- associated molecule, that mediates apoptosis (A.M. Chinnaiyan *et al.*,
Cell 81: 505-12 (1995); M.P. Boldin *et al.*, *J. Biol Chem 270*:7795-8 (1995); F.C.
Kischkel *et al.*, *EMBO 14*: 5579-5588 (1995)). Thus, the inventors demonstrate
that RIP, TRAF2 and FADD could be co-immunoprecipitated with DR3. In 293
cells expressing DR3 and RIP, only a weak association could be detected between

the two molecules. However, in the presence of TRADD, RIP association with DR3 was significantly enhanced. Likewise, very little TRAF2 directly co-precipitated with DR3 in 293 cells. However, when DR3 and TRAF2 were expressed in the presence of TRADD and RIP (both of which can bind TRAF2), an enhanced binding of TRAF2 to DR3 could be detected. A similar association between FADD and DR3 was also observed. In the presence of TRADD, FADD efficiently coprecipitated with DR3.

Previous studies demonstrated that FADD could recruit the ICE/CED-3-like protease FLICE to the Fas/APO-1 death inducing signaling complex (M. Muzio *et al.*, *Cell* 85: 817-827 (1996); M.P. Boldin *et al.*, *Cell* 85: 803-815 (1996)). To demonstrate that FLICE can associate with TNFR-1 and DR3, coprecipitation experiments in 293 cells were carried out. Interestingly, FLICE was found complexed to TNFR-1 and DR3. Co-transfection of TRADD and/or FADD failed to enhance the FLICE-TNFR-1/DR3 interaction, suggesting that endogenous amounts of these adapter molecules were sufficient to maintain this association.

Example 6

DR3 Induced Apoptosis and NF- κ B Activation

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio *et al.*, *Cell* 85: 817-827 (1996); M. P. Boldin *et al.*, *Cell* 85: 803-815 (1996)). Thus, this system was utilized to study the functional role of DDCR. Ectopic expression of DR3 in MCF7 breast carcinoma cells and 293 human embryonic kidney cells induced rapid apoptosis.

Experimental Design

Cell death assays were performed essentially as previously described (A.M. Chinnaiyan *et al.*, *Cell* 81: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem* 270:

7795-8 (1995); F.C. Kischkel *et al.*, *EMBO 14*: 5579-5588 (1995); A.M. Chinnaiyan *et al.*, *J Biol Chem 271*: 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines stably transfected with either vector alone, a CrmA expression construct (M. Tewari *et al.*, *J Biol Chem 270*: 3255-60 (1995)), or FADD-DN expression construct (A.M. Chinnaiyan *et al.*, *J Biol Chem 271*: 4961-4965 (1996)) were transiently transfected with pCMV- β -galactosidase in the presence of a ten-fold excess of pcDNA3 expression constructs encoding the indicated proteins using lipofectamine (GIBCO-BRL). 293 cells were likewise transfected using the CaPO₄ method. The ICE family inhibitor z-VAD-fmk (Enzyme Systems Products, Dublin, CA) was added to the cells at a concentration of 10 μ M, 5 hrs after transfection. 32 hours following transfection, cells were fixed and stained with X-Gal as previously described (A.M. Chinnaiyan *et al.*, *Cell 81*: 505-12 (1995); M.P. Boldin *et al.*, *J Biol Chem 270*: 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO 14*: 5579-5588 (1995)). The data (mean \pm SD) shown are the percentage of round blue cells among the total number of blue cells counted. Data were obtained from at least three independent experiments.

NF- κ B luciferase assays were performed as described elsewhere (H. Hsu *et al.*, *Immunity 4*: 387-396 (1996); M.D. Adams *et al.*, *Nature 377*: 3-174 (1995); G.S. Feng *et al.*, *J Biol Chem 271*: 12129-32 (1996); M. Rothe *et al.*, *Cell 78*: 681-92 (1994); M. Rothe *et al.*, *Science 269*:1424-1427 (1995); A.M. Chinnaiyan *et al.*, *J Biol Chem 271*: 4961-4965 (1996)). Briefly, 293 cells were co-transfected by calcium phosphate precipitation with pCMV- β -galactosidase, E-selectin-luciferase reporter gene (M. Rothe *et al.*, *Cell 78*: 681-92 (1994); M. Rothe *et al.*, *Science 269*:1424-1427 (1995)), the indicated death receptors, and the indicated dominant negative inhibitors. In addition, DR3 or DDR3 was cotransfected with the pLantern expression construct (GIBCO-BRL) which encodes green fluorescent protein (photographic inset). Cells were visualized by fluorescence microscopy using a FITC range barrier filter cube. Nuclei of transfected cells were visualized by DAPI staining and the image overlaid. (Cell

death assays were performed essentially as previously described (Chinnaiyan *et al.*, *Cell* 81:505-12 (1995); Boldin, *et al.*, *J. Biol. Chem.* 270:7795-8 (1995); Kischkel *et al.*, *EMBO* 14:5579-5588 (1995)); Chinnaiyan *et al.*, *J. Biol. Chem.* 271:4961-4965 (1996)). The dominant negative inhibitors were used at a 4-fold higher quantity than the death receptors. Total DNA was kept constant.

To show that DR3 induces NF- κ B activation which is inhibitable by RIP-DN (Stanger *et al.*, *Cell* 81:513-23 (1995)) and TRAF2-DN (Hsu *et al.*, *Cell* 81:495-504 (1995); Rothe *et al.*, *Cell* 78:681-92 (1994); Rothe *et al.* *Science* 269:1424-1427 (1995)), 293 cells were co-transfected with the indicated molecules and an NF- κ B luciferase reporter plasmid (Rothe *et al.*, *Cell* 78:681-92 (1994); Rothe *et al.*, *Science* 269:1424-1427 (1995)), and luciferase activities subsequently determined. NF- κ B luciferase assays were performed as described elsewhere (Hsu *et al.*, *Immunity* 4:387-396 (1996); Adams *et al.*, *Nature* 377:3-174 (1995); Feng *et al.*, *J. Biol. Chem.* 271:12129-32 (1996); Rothe *et al.*, *Cell* 78:681-92 (1994); Rothe *et al.* *Science* 269:1424-1427 (1995); Chinnaiyan *et al.*, *J. Biol. Chem.* 271:4961-4965 (1996)). Briefly, 293 cells were co-transfected by calcium phosphate precipitation with pCMB- β -galactosidase, E-selectin-luciferase reporter gene (Rothe *et al.*, *Cell* 78:681-92 (1994); Rothe *et al.*, *Science* 269:1424-1427 (1995)), the indicated death receptors, and the indicated dominant negative inhibitors. The dominant negative inhibitors were used at a 4-fold higher quantity than the death receptors. Total DNA was kept constant. Representative experiment performed in duplicate three independent times (mean \pm SD).

Results

The cells displayed morphological alterations typical of cells undergoing apoptosis, becoming rounded, condensed and detaching from the dish. In MCF7 cells, plasmids encoding full-length DR3 or DDR3 were co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR3, but not DDR3, exhibited apoptotic morphology as assessed

by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio *et al.*, *Cell* 85: 817-827 (1996); M. P. Boldin *et al.*, *Cell* 85: 803-815 (1996); M. Tewari *et al.*, *J Biol Chem* 270: 3255-60 (1995)), DR3-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. Importantly, apoptosis induced by DR3 was also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S), which were previously shown to inhibit death signaling by Fas/APO-1 and TNFR-1 (M. Muzio *et al.*, *Cell* 85: 817-827 (1996); M. P. Boldin *et al.*, *Cell* 85: 803-815 (1996); H. Hsu *et al.*, *Cell* 84: 299-398 (1996); A.M. Chinnaiyan *et al.*, *J Biol Chem* 271: 4961-4965 (1996)). Thus, FADD and the ICE-like protease FLICE are likely necessary components of DR3-induced apoptosis.

As DR3 activation recruits three molecules implicated in TNF-induced NF-kB activation, we examined whether DR3 could activate NF-kB. Transfection of a control vector or expression of Fas/APO-1 failed to induce NF-kB activation. By contrast, NF-kB was activated by ectopic expression of DR3 or TNFR-1, but not by the inactive signaling mutants DDR3 or DTNFR-1. Importantly, DR3-induced NF-kB activation was blocked by dominant negative derivatives of RIP (RIP-DN) and TRAF2 (TRAF2-DN), which were previously shown to abrogate TNF-induced NF-kB activation (H. Hsu *et al.*, *Cell* 84: 299-398 (1996); H. Hsu *et al.*, *Immunity* 4: 387-396 (1996)). As expected, FADD-DN did not interfere with DR3-mediated NF-kB activation (H. Hsu *et al.*, *Cell* 84: 299-398 (1996); A.M. Chinnaiyan *et al.*, *J Biol Chem* 271: 4961-4965 (1996)).

Thus, the experiments set forth in Examples 6 and 7 demonstrate that DR3 is a death domain-containing molecule capable of triggering both apoptosis and NF-kB activation, two pathways dominant in the regulation of the immune system. The experiments also demonstrate the internal signal transduction machinery of this novel cell death receptor. The DR3 signaling complex assembles in a hierarchical manner with the recruitment of the multivalent adapter molecule TRADD, from which two distinct signaling cascades emanate: 1) NF-kB

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

The entire disclosures of all patents, patent applications, and publications referred to herein are hereby incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Yu, Guo-Liang
Ni, Jian
Dixit, Vishva
Gentz, Reiner L.
Dillon, Patrick J.

(ii) TITLE OF INVENTION: Death Domain Containing Receptors

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: 1100 New York Ave., NW, Suite 600
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: USA
(F) ZIP: 20005-3934

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: HERewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: Not Yet Assigned
(B) FILING DATE: 06-FEB-1997

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/028,711
(B) FILING DATE: 17-OCT-1996

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/013,285
(B) FILING DATE: 12-MAR-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Steffe, Eric K.
(B) REGISTRATION NUMBER: 36,688
(C) REFERENCE/DOCKET NUMBER: 1488.0310003/EKS/KRM

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-371-2600
(B) TELEFAX: 202-371-2540

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1783 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 198..1481

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATGGGTGGG GGTGGGGGCG CTGCTGGATT CCTGCTCTGG TGGAGGGGAA ACTTGTGAGG	60
GGCTGGTAAG CGCCCCCTCC GAAGCCTGGT GTGTGCGCGG GGGGAAGGAA GTTAGTTTCC	120
TCTCCACCCA TGGGCACCCC TTCTGCCCGG GGCCTGGGAA GTGGGCTGCT CTGTGGGCAA	180
ATGCTGGGGC CTCTGAA ATG GAG GAG ACG CAG CAG GGA GAG GCC CCA CGT	230
Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg	
1 5 10	
GGG CAG CTG CGC GGA GAG TCA GCA GCA CCT GTC CCC CAG GCG CTC CTC	278
Gly Gln Leu Arg Gly Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu	
15 20 25	
CTG GTG CTG CTG GGG GCC CGG GCC CAG GGC GGC ACT CGT AGC CCC AGG	326
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg	
30 35 40	
TGT GAC TGT GCC GGT GAC TTC CAC AAG AAG ATT GGT CTG TTT TGT TGC	374
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys	
45 50 55	
AGA GGC TGC CCA GCG GGG CAC TAC CTG AAG GCC CCT TGC ACG GAG CCC	422
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro	
60 65 70 75	
TGC GGC AAC TCC ACC TGC CTT GTG TGT CCC CAA GAC ACC TTC TTG GCC	470
Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala	
80 85 90	
TGG GAG AAC CAC CAT AAT TCT GAA TGT GCC CGC TGC CAG GCC TGT GAT	518
Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp	
95 100 105	
GAG CAG GCC TCC CAG GTG GCG CTG GAG AAC TGT TCA GCA GTG GCC GAC	566
Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp	
110 115 120	

66450-43560

ACC CGC TGT GGC TGT AAG CCA GGC TGG TTT GTG GAG TGC CAG GTC AGC	614
Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser	
125 130 135	
CAA TGT GTC AGC AGT TCA CCC TTC TAC TGC CAA CCA TGC CTA GAC TGC	662
Gln Cys Val Ser Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys	
140 145 150 155	
GGG GCC CTG CAC CGC CAC ACA CGG CTA CTC TGT TCC CGC AGA GAT ACT	710
Gly Ala Leu His Arg His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr	
160 165 170	
GAC TGT GGG ACC TGC CTG CCT GGC TTC TAT GAA CAT GGC GAT GGC TGC	758
Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys	
175 180 185	
GTG TCC TGC CCC ACG AGC ACC CTG GGG AGC TGT CCA GAG CGC TGT GCC	806
Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala	
190 195 200	
GCT GTC TGT GGC TGG AGG CAG ATG TTC TGG GTC CAG GTG CTC CTG GCT	854
Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala	
205 210 215	
GGC CTT GTG GTC CCC CTC CTG CTT GGG GCC ACC CTG ACC TAC ACA TAC	902
Gly Leu Val Val Pro Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr	
220 225 230 235	
CGC CAC TGC TGG CCT CAC AAG CCC CTG GTT ACT GCA GAT GAA GCT GGG	950
Arg His Cys Trp Pro His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly	
240 245 250	
ATG GAG GCT CTG ACC CCA CCA CCG GCC ACC CAT CTG TCA CCC TTG GAC	998
Met Glu Ala Leu Thr Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp	
255 260 265	
AGC GCC CAC ACC CTT CTA GCA CCT CCT GAC AGC AGT GAG AAG ATC TGC	1046
Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys	
270 275 280	
ACC GTC CAG TTG GTG GGT AAC AGC TGG ACC CCT GGC TAC CCC GAG ACC	1094
Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr	
285 290 295	
CAG GAG GCG CTC TGC CCG CAG GTG ACA TGG TCC TGG GAC CAG TTG CCC	1142
Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro	
300 305 310 315	
AGC AGA GCT CTT GGC CCC GCT GCT GCG CCC ACA CTC TCG CCA GAG TCC	1190
Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser	
320 325 330	
CCA GCC GGC TCG CCA GCC ATG ATG CTG CAG CCG GGC CCG CAG CTC TAC	1238
Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr	
335 340 345	

GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC	1286
Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg	
350 355 360	
ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC	1334
Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile	
365 370 375	
GGC CGC TTC CGA GAC CAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG	1382
Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln	
380 385 390 395	
CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG	1430
Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met	
400 405 410	
GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC	1478
Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly	
415 420 425	
CCG TGACACGGCG CCCACTTGCC ACCTAGGCGC TCTGGTGGCC CTTGCAGAAG	1531
Pro	
CCCTAAGTAC GGTTACTTAT GCGTGTAGAC ATTTTATGTC ACTTATTAAG CCGCTGGCAC	1591
GGCCCTGCGT AGCAGCACCA GCCGGCCCCA CCCCTGCTCG CCCCTATCGC TCCAGCCAAG	1651
GCGAAGAAGC ACGAACGAAT GTCGAGAGGG GGTGAAGACA TTTCTCAACT TCTCGGCCGG	1711
AGTTTGGCTG AGATCGCGGT ATTAAATCTG TGAAAGAAAA CAAAACAAAA CAAAAAAAAA	1771
AAAAAAAAAA AA	1783

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 428 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg Gly Gln Leu Arg Gly	
1 5 10 15	
Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu Leu Val Leu Leu Gly	
20 25 30	
Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg Cys Asp Cys Ala Gly	
35 40 45	

Asp	Phe	His	Lys	Lys	Ile	Gly	Leu	Phe	Cys	Cys	Arg	Gly	Cys	Pro	Ala	
50						55					60					
Gly	His	Tyr	Leu	Lys	Ala	Pro	Cys	Thr	Glu	Pro	Cys	Gly	Asn	Ser	Thr	
65					70					75					80	
Cys	Leu	Val	Cys	Pro	Gln	Asp	Thr	Phe	Leu	Ala	Trp	Glu	Asn	His	His	
				85					90					95		
Asn	Ser	Glu	Cys	Ala	Arg	Cys	Gln	Ala	Cys	Asp	Glu	Gln	Ala	Ser	Gln	
			100					105					110			
Val	Ala	Leu	Glu	Asn	Cys	Ser	Ala	Val	Ala	Asp	Thr	Arg	Cys	Gly	Cys	
			115				120					125				
Lys	Pro	Gly	Trp	Phe	Val	Glu	Cys	Gln	Val	Ser	Gln	Cys	Val	Ser	Ser	
130						135					140					
Ser	Pro	Phe	Tyr	Cys	Gln	Pro	Cys	Leu	Asp	Cys	Gly	Ala	Leu	His	Arg	
145					150					155					160	
His	Thr	Arg	Leu	Leu	Cys	Ser	Arg	Arg	Asp	Thr	Asp	Cys	Gly	Thr	Cys	
				165					170					175		
Leu	Pro	Gly	Phe	Tyr	Glu	His	Gly	Asp	Gly	Cys	Val	Ser	Cys	Pro	Thr	
			180					185					190			
Ser	Thr	Leu	Gly	Ser	Cys	Pro	Glu	Arg	Cys	Ala	Ala	Val	Cys	Gly	Trp	
195						200					205					
Arg	Gln	Met	Phe	Trp	Val	Gln	Val	Leu	Leu	Ala	Gly	Leu	Val	Val	Pro	
210						215					220					
Leu	Leu	Leu	Gly	Ala	Thr	Leu	Thr	Tyr	Thr	Tyr	Arg	His	Cys	Trp	Pro	
225					230					235					240	
His	Lys	Pro	Leu	Val	Thr	Ala	Asp	Glu	Ala	Gly	Met	Glu	Ala	Leu	Thr	
				245					250					255		
Pro	Pro	Pro	Ala	Thr	His	Leu	Ser	Pro	Leu	Asp	Ser	Ala	His	Thr	Leu	
			260					265					270			
Leu	Ala	Pro	Pro	Asp	Ser	Ser	Glu	Lys	Ile	Cys	Thr	Val	Gln	Leu	Val	
275							280					285				
Gly	Asn	Ser	Trp	Thr	Pro	Gly	Tyr	Pro	Glu	Thr	Gln	Glu	Ala	Leu	Cys	
290						295					300					
Pro	Gln	Val	Thr	Trp	Ser	Trp	Asp	Gln	Leu	Pro	Ser	Arg	Ala	Leu	Gly	
305					310					315					320	
Pro	Ala	Ala	Ala	Pro	Thr	Leu	Ser	Pro	Glu	Ser	Pro	Ala	Gly	Ser	Pro	
			325					330					335			
Ala	Met	Met	Leu	Gln	Pro	Gly	Pro	Gln	Leu	Tyr	Asp	Val	Met	Asp	Ala	

340	345	350
Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg Thr Leu Gly Leu Arg		
355	360	365
Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile Gly Arg Phe Arg Asp		
370	375	380
Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln Gln Gln Pro Ala Gly		
385	390	395
Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met Gly Leu Asp Gly Cys		
405	410	415
Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly Pro		
420	425	

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1254 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1251

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GAG CAG CGG CCG CGG GGC TGC GCG GCG GTG GCG GCG GCG CTC CTC	48
Met Glu Gln Arg Pro Arg Gly Cys Ala Ala Val Ala Ala Leu Leu	
430	435
CTG GTG CTG CTG GGG GCC CGG GCC CAG GGC GGC ACT CGT AGC CCC AGG	96
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg	
445	450
TGT GAC TGT GCC GGT GAC TTC CAC AAG AAG ATT GGT CTG TTT TGT TGC	144
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys	
465	470
AGA GGC TGC CCA GCG GGG CAC TAC CTG AAG GCC CCT TGC ACG GAG CCC	192
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro	
480	485
TGC GGC AAC TCC ACC TGC CTT GTG TGT CCC CAA GAC ACC TTC TTG GCC	240
Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala	
495	500

TGG	GAG	AAC	CAC	CAT	AAT	TCT	GAA	TGT	GCC	CGC	TGC	CAG	GCC	TGT	GAT	288
Trp	Glu	Asn	His	His	Asn	Ser	Glu	Cys	Ala	Arg	Cys	Gln	Ala	Cys	Asp	
510						515			520							
GAG	CAG	GCC	TCC	CAG	GTG	GCG	CTG	GAG	AAC	TGT	TCA	GCA	GTG	GCC	GAC	336
Glu	Gln	Ala	Ser	Gln	Val	Ala	Leu	Glu	Asn	Cys	Ser	Ala	Val	Ala	Asp	
525			530			535			540							
ACC	CGC	TGT	GGC	TGT	AAG	CCA	GGC	TGG	TTT	GTG	GAG	TGC	CAG	GTC	AGC	384
Thr	Arg	Cys	Gly	Cys	Lys	Pro	Gly	Trp	Phe	Val	Glu	Cys	Gln	Val	Ser	
			545			550			555							
CAA	TGT	GTC	AGC	AGT	TCA	CCC	TTC	TAC	TGC	CAA	CCA	TGC	CTA	GAC	TGC	432
Gln	Cys	Val	Ser	Ser	Ser	Pro	Phe	Tyr	Cys	Gln	Pro	Cys	Leu	Asp	Cys	
			560			565			570							
GGG	GCC	CTG	CAC	CGC	CAC	ACA	CGG	CTA	CTC	TGT	TCC	CGC	AGA	GAT	ACT	480
Gly	Ala	Leu	His	Arg	His	Thr	Arg	Leu	Leu	Cys	Ser	Arg	Arg	Asp	Thr	
575			580			585			590							
GAC	TGT	GGG	ACC	TGC	CTG	CCT	GGC	TTC	TAT	GAA	CAT	GGC	GAT	GGC	TGC	528
Asp	Cys	Gly	Thr	Cys	Leu	Pro	Gly	Phe	Tyr	Glu	His	Gly	Asp	Gly	Cys	
590			595			600			605							
GTG	TCC	TGC	CCC	ACG	AGC	ACC	CTG	GGG	AGC	TGT	CCA	GAG	CGC	TGT	GCC	576
Val	Ser	Cys	Pro	Thr	Ser	Thr	Leu	Gly	Ser	Cys	Pro	Glu	Arg	Cys	Ala	
605			610			615			620							
GCT	GTC	TGT	GGC	TGG	AGG	CAG	ATG	TTC	TGG	GTC	CAG	GTG	CTC	CTG	GCT	624
Ala	Val	Cys	Gly	Trp	Arg	Gln	Met	Phe	Trp	Val	Gln	Val	Leu	Leu	Ala	
			625			630			635							
GGC	CTT	GTG	GTC	CCC	CTC	CTG	CTT	GGG	GCC	ACC	CTG	ACC	TAC	ACA	TAC	672
Gly	Leu	Val	Val	Pro	Leu	Leu	Leu	Gly	Ala	Thr	Leu	Thr	Tyr	Thr	Tyr	
640			645			650			655							
CGC	CAC	TGC	TGG	CCT	CAC	AAG	CCC	CTG	GTT	ACT	GCA	GAT	GAA	GCT	GGG	720
Arg	His	Cys	Trp	Pro	His	Lys	Pro	Leu	Val	Thr	Ala	Asp	Glu	Ala	Gly	
655			660			665			670							
ATG	GAG	GCT	CTG	ACC	CCA	CCA	CCG	GCC	ACC	CAT	CTG	TCA	CCC	TTG	GAC	768
Met	Glu	Ala	Leu	Thr	Pro	Pro	Pro	Ala	Thr	His	Leu	Ser	Pro	Leu	Asp	
670			675			680			685							
AGC	GCC	CAC	ACC	CTT	CTA	GCA	CCT	CCT	GAC	AGC	AGT	GAG	AAG	ATC	TGC	816
Ser	Ala	His	Thr	Leu	Leu	Ala	Pro	Pro	Asp	Ser	Ser	Glu	Lys	Ile	Cys	
685			690			695			700							
ACC	GTC	CAG	TTG	GTG	GGT	AAC	AGC	TGG	ACC	CCT	GGC	TAC	CCC	GAG	ACC	864
Thr	Val	Gln	Leu	Val	Gly	Asn	Ser	Trp	Thr	Pro	Gly	Tyr	Pro	Glu	Thr	
			705			710			715							
CAG	GAG	GCG	CTC	TGC	CCG	CAG	GTG	ACA	TGG	TCC	TGG	GAC	CAG	TTG	CCC	912
Gln	Glu	Ala	Leu	Cys	Pro	Gln	Val	Thr	Trp	Ser	Trp	Asp	Gln	Leu	Pro	
720			725			730			735							

AGC AGA GCT CTT GGC CCC GCT GCT GCG CCC ACA CTC TCG CCA GAG TCC	960
Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser	
735 740 745	
CCA GCC GGC TCG CCA GCC ATG ATG CTG CAG CCG GGC CCG CAG CTC TAC	1008
Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr	
750 755 760	
GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC	1056
Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg	
765 770 775 780	
ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC	1104
Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile	
785 790 795	
GGC CGC TTC CGA GAC CAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG	1152
Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln	
800 805 810	
CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG	1200
Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met	
815 820 825	
GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC	1248
Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly	
830 835 840	
CCG TGA	1254
Pro	
845	

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Gln Arg Pro Arg Gly Cys Ala Ala Val Ala Ala Ala Leu Leu	
1 5 10 15	
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg	
20 25 30	
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys	
35 40 45	
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro	
50 55 60	

Cys 65	Gly	Asn	Ser	Thr	Cys 70	Leu	Val	Cys	Pro	Gln	Asp	Thr	Phe	Leu	Ala 80
Trp	Glu	Asn	His	His 85	Asn	Ser	Glu	Cys	Ala 90	Arg	Cys	Gln	Ala	Cys 95	Asp
Glu	Gln	Ala	Ser 100	Gln	Val	Ala	Leu	Glu	Asn 105	Cys	Ser	Ala	Val	Ala 110	Asp
Thr	Arg	Cys 115	Gly	Cys	Lys	Pro	Gly 120	Trp	Phe	Val	Glu	Cys 125	Gln	Val	Ser
Gln	Cys 130	Val	Ser	Ser	Ser	Pro 135	Phe	Tyr	Cys	Gln	Pro 140	Cys	Leu	Asp	Cys
Gly 145	Ala	Leu	His	Arg	His 150	Thr	Arg	Leu	Leu	Cys 155	Ser	Arg	Arg	Asp	Thr 160
Asp	Cys	Gly	Thr	Cys 165	Leu	Pro	Gly	Phe	Tyr 170	Glu	His	Gly	Asp	Gly 175	Cys
Val	Ser	Cys	Pro 180	Thr	Ser	Thr	Leu	Gly 185	Ser	Cys	Pro	Glu	Arg	Cys 190	Ala
Ala	Val	Cys 195	Gly	Trp	Arg	Gln	Met 200	Phe	Trp	Val	Gln	Val 205	Leu	Leu	Ala
Gly 210	Leu	Val	Val	Pro	Leu	Leu 215	Leu	Gly	Ala	Thr	Leu 220	Thr	Tyr	Thr	Tyr
Arg 225	His	Cys	Trp	Pro	His 230	Lys	Pro	Leu	Val	Thr 235	Ala	Asp	Glu	Ala	Gly 240
Met	Glu	Ala	Leu	Thr 245	Pro	Pro	Pro	Ala	Thr 250	His	Leu	Ser	Pro	Leu 255	Asp
Ser	Ala	His	Thr 260	Leu	Leu	Ala	Pro	Pro 265	Asp	Ser	Ser	Glu	Lys 270	Ile	Cys
Thr	Val	Gln 275	Leu	Val	Gly	Asn	Ser 280	Trp	Thr	Pro	Gly	Tyr 285	Pro	Glu	Thr
Gln 290	Glu	Ala	Leu	Cys	Pro	Gln 295	Val	Thr	Trp	Ser	Trp 300	Asp	Gln	Leu	Pro
Ser 305	Arg	Ala	Leu	Gly	Pro	Ala 310	Ala	Ala	Pro	Thr 315	Leu	Ser	Pro	Glu	Ser 320
Pro	Ala	Gly	Ser	Pro 325	Ala	Met	Met	Leu	Gln 330	Pro	Gly	Pro	Gln	Leu 335	Tyr
Asp	Val	Met	Asp 340	Ala	Val	Pro	Ala	Arg 345	Arg	Trp	Lys	Glu	Phe 350	Val	Arg
Thr	Leu	Gly	Leu	Arg	Glu	Ala	Glu	Ile	Glu	Ala	Val	Glu	Val	Glu	Ile

Pro

(i) SEQUENCE CHARACTERISTICS:

- (ii) MOLECULE TYPE: protein

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
20 25 30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn	Cys	Ser	Leu	Cys	Leu	Asn	Gly	Thr	Val	His	Leu	Ser	Cys	Gln	Glu	145	150	155	160
Lys	Gln	Asn	Thr	Val	Cys	Thr	Cys	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu	165	170	175	
Asn	Glu	Cys	Val	Ser	Cys	Ser	Asn	Cys	Lys	Lys	Ser	Leu	Glu	Cys	Thr	180	185	190	
Lys	Leu	Cys	Leu	Pro	Gln	Ile	Glu	Asn	Val	Lys	Gly	Thr	Glu	Asp	Ser	195	200	205	
Gly	Thr	Thr	Val	Leu	Leu	Pro	Leu	Val	Ile	Phe	Phe	Gly	Leu	Cys	Leu	210	215	220	
Leu	Ser	Leu	Leu	Phe	Ile	Gly	Leu	Met	Tyr	Arg	Tyr	Gln	Arg	Trp	Lys	225	230	235	240
Ser	Lys	Leu	Tyr	Ser	Ile	Val	Cys	Gly	Lys	Ser	Thr	Pro	Glu	Lys	Glu	245	250	255	
Gly	Glu	Leu	Glu	Gly	Thr	Thr	Thr	Lys	Pro	Leu	Ala	Pro	Asn	Pro	Ser	260	265	270	
Phe	Ser	Pro	Thr	Pro	Gly	Phe	Thr	Pro	Thr	Leu	Gly	Phe	Ser	Pro	Val	275	280	285	
Pro	Ser	Ser	Thr	Phe	Thr	Ser	Ser	Ser	Thr	Tyr	Thr	Pro	Gly	Asp	Cys	290	295	300	
Pro	Asn	Phe	Ala	Ala	Pro	Arg	Arg	Glu	Val	Ala	Pro	Pro	Tyr	Gln	Gly	305	310	315	320
Ala	Asp	Pro	Ile	Leu	Ala	Thr	Ala	Leu	Ala	Ser	Asp	Pro	Ile	Pro	Asn	325	330	335	
Pro	Leu	Gln	Lys	Trp	Glu	Asp	Ser	Ala	His	Lys	Pro	Gln	Ser	Leu	Asp	340	345	350	
Thr	Asp	Asp	Pro	Ala	Thr	Leu	Tyr	Ala	Val	Val	Glu	Asn	Val	Pro	Pro	355	360	365	
Leu	Arg	Trp	Lys	Glu	Phe	Val	Arg	Arg	Leu	Gly	Leu	Ser	Asp	His	Glu	370	375	380	
Ile	Asp	Arg	Leu	Glu	Leu	Gln	Asn	Gly	Arg	Cys	Leu	Arg	Glu	Ala	Gln	385	390	395	400
Tyr	Ser	Met	Leu	Ala	Thr	Trp	Arg	Arg	Arg	Thr	Pro	Arg	Arg	Glu	Ala	405	410	415	
Thr	Leu	Glu	Leu	Leu	Gly	Arg	Val	Leu	Arg	Asp	Met	Asp	Leu	Leu	Gly	420	425	430	
Cys	Leu	Glu	Asp	Ile	Glu	Glu	Ala	Leu	Cys	Gly	Pro	Ala	Ala	Leu	Pro				

435

440

445

Pro Ala Pro Ser Leu Leu Arg
450 455

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 335 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Leu	Gly	Ile	Trp	Thr	Leu	Leu	Pro	Leu	Val	Leu	Thr	Ser	Val	Ala
1				5					10					15	
Arg	Leu	Ser	Ser	Lys	Ser	Val	Asn	Ala	Gln	Val	Thr	Asp	Ile	Asn	Ser
			20					25						30	
Lys	Gly	Leu	Glu	Leu	Arg	Lys	Thr	Val	Thr	Thr	Val	Glu	Thr	Gln	Asn
		35					40					45			
Leu	Glu	Gly	Leu	His	His	Asp	Gly	Gln	Phe	Cys	His	Lys	Pro	Cys	Pro
	50					55					60				
Pro	Gly	Glu	Arg	Lys	Ala	Arg	Asp	Cys	Thr	Val	Asn	Gly	Asp	Glu	Pro
65				70						75				80	
Asp	Cys	Val	Pro	Cys	Gln	Glu	Gly	Lys	Glu	Tyr	Thr	Asp	Lys	Ala	His
				85					90					95	
Phe	Ser	Ser	Lys	Cys	Arg	Arg	Cys	Arg	Leu	Cys	Asp	Glu	Gly	His	Gly
			100					105						110	
Leu	Glu	Val	Glu	Ile	Asn	Cys	Thr	Arg	Thr	Gln	Asn	Thr	Lys	Cys	Arg
		115					120					125			
Cys	Lys	Pro	Asn	Phe	Phe	Gln	Asn	Ser	Thr	Val	Cys	Glu	His	Cys	Asp
	130						135					140			
Pro	Cys	Thr	Lys	Cys	Glu	His	Gly	Ile	Ile	Lys	Glu	Cys	Thr	Leu	Thr
145					150					155				160	
Ser	Asn	Thr	Lys	Cys	Lys	Glu	Glu	Gly	Ser	Arg	Ser	Asn	Leu	Gly	Trp
				165					170					175	
Leu	Cys	Leu	Leu	Leu	Leu	Pro	Ile	Pro	Leu	Ile	Val	Trp	Val	Lys	Arg
			180					185					190		

Lys	Glu	Val	Gln	Lys	Thr	Cys	Arg	Lys	His	Arg	Lys	Glu	Asn	Gln	Gly
	195						200					205			
Ser	His	Glu	Ser	Pro	Thr	Leu	Asn	Pro	Glu	Thr	Val	Ala	Ile	Asn	Leu
	210					215					220				
Ser	Asp	Val	Asp	Leu	Ser	Lys	Tyr	Ile	Thr	Thr	Ile	Ala	Gly	Val	Met
225					230				235						240
Thr	Leu	Ser	Gln	Val	Lys	Gly	Phe	Val	Arg	Lys	Asn	Gly	Val	Asn	Glu
			245						250					255	
Ala	Lys	Ile	Asp	Glu	Ile	Lys	Asn	Asp	Asn	Val	Gln	Asp	Thr	Ala	Glu
			260					265					270		
Gln	Lys	Val	Gln	Leu	Leu	Arg	Asn	Trp	His	Gln	Leu	His	Gly	Lys	Lys
		275					280					285			
Glu	Ala	Tyr	Asp	Thr	Leu	Ile	Lys	Asp	Leu	Lys	Lys	Ala	Asn	Leu	Cys
	290					295					300				
Thr	Leu	Ala	Glu	Lys	Ile	Gln	Thr	Ile	Ile	Leu	Lys	Asp	Ile	Thr	Ser
305					310					315					320
Asp	Ser	Glu	Asn	Ser	Asn	Phe	Arg	Asn	Glu	Ile	Gln	Ser	Leu	Val	
			325					330						335	

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGCCATGGG GGCCCGGCGG CAG

23

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

559199 995555

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGAAGCTTC TAGGACCCAG AACATCTGCC

30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCGTCTAGAT CAAAGCGTAG TCTGGGACGT CGTATGGGTA CGGGCCGCGC TGCA

54

55449099660

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGCGGATCCT CACGGGCCGC GCTGCA

26

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGAGATCTA GTCTGGACCC AGAACATCTG CCTCC

35

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

What Is Claimed Is:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

5 (a) a nucleotide sequence encoding the full-length death domain containing receptor polypeptide (DR3-V1) having the complete amino acid sequence in Figure 1 (SEQ ID NO:2);

10 (b) nucleotide sequence encoding the full-length death domain containing receptor (DR3) polypeptide having the complete amino acid sequence in Figure 2 (SEQ ID NO:4), including the predicted leader sequence;

(c) a nucleotide sequence encoding the DR3-V1 polypeptide having the amino acid sequence at positions from about 36 to about 428 in Figure 1 (SEQ ID NO:2);

15 (d) a nucleotide sequence encoding the full-length DR3-V1 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97456;

(e) a nucleotide sequence encoding the full-length DR3 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97757;

20 (f) a nucleotide sequence encoding the mature DR3-V1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456;

25 (g) a nucleotide sequence encoding the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97757;

(h) a nucleotide sequence that encodes the DR3 extracellular domain;

- 5 (i) a nucleotide sequence that encodes the DR3 transmembrane domain;
- (j) a nucleotide sequence that encodes the DR3 intracellular domain;
- (k) a nucleotide sequence that encodes the DR3 death domain;
- and
- (l) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k).

10 2. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figure 1 (SEQ ID NO:1).

15 3. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the death domain containing receptor polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2).

20 4. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the mature death domain containing receptor polypeptide having the amino acid sequence in Figure 1 (SEQ ID NO:2).

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97456.

6. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figure 2 (SEQ ID NO:3).

7. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the death domain containing receptor polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2).

5 8. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 2 (SEQ ID NO:3) encoding the mature death domain containing receptor polypeptide having the amino acid sequence in Figure 2 (SEQ ID NO:4).

10 9. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No.97757.

15 10. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the death domain containing receptor polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.

11. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the mature death domain containing receptor polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.

20 12. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the death domain containing receptor polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97757.

13. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the mature death domain containing receptor polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97757.

14. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j), (k) or (l) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

15. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a death domain containing receptor polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k) of claim 1.

16. The isolated nucleic acid molecule of claim 15, which encodes an epitope-bearing portion of a death domain containing receptor polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from

about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in Figure 1 (SEQ ID NO:2).

5 17. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

18. A recombinant vector produced by the method of claim 17.

10 19. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 18 into a host cell.

20. A recombinant host cell produced by the method of claim 19.

15 21. A recombinant method for producing a death domain containing receptor polypeptide, comprising culturing the recombinant host cell of claim 20 under conditions such that said polypeptide is expressed and recovering said polypeptide.

22. An isolated death domain containing receptor polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

20 (a) the amino acid sequence of the DR3-V1 polypeptide having the complete 428 amino acid sequence, including the leader sequence shown in Figure 1 (SEQ ID NO:2);

 (b) the amino acid sequence of the DR3 polypeptide having the complete 417 amino acid sequence, including the leader sequence, shown in Figure 2 (SEQ ID NO:4);

(c) the amino acid sequence of the DR3-V1 polypeptide having the amino acid sequence at positions from about 36 to about 428 in Figure 1 (SEQ ID NO:2);

5 (d) the amino acid sequence of the DR3-V1 polypeptide having the complete amino acid sequence, including the leader, encoded by the cDNA clone contained in ATCC Deposit No. 97456;

(e) the amino acid sequence of the DR3 polypeptide having the complete amino acid sequence, including the leader, encoded by the cDNA clone contained in ATCC Deposit No. 97757;

10 (f) the amino acid sequence of the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456;

15 (g) the amino acid sequence encoding the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97757;

(h) the amino acid sequence of the DR3 extracellular domain;

(i) the amino acid sequence of the DR3 transmembrane domain;

(j) the amino acid sequence of the DR3 intracellular domain;

20 (k) the amino acid sequence of the DR3 death domain; and

(l) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k).

23. An isolated antibody that binds specifically to a death domain containing receptor polypeptide of claim 22.

25 24. A method of treating diseases and disorders associated with the inhibition of apoptosis comprising administering an effective amount of the

5

25. A method of treating diseases and disorders associated with increased apoptosis comprising administering to a patient in need thereof an effective amount of an antagonist of the polypeptide as claimed in claim 22 to a patient in need thereof.

26. A method of treating inflammatory diseases and disorders comprising administering to a patient in need thereof an effective amount of an agent consisting of the polypeptide as claimed in claim 22.

Death Domain Containing Receptors

Abstract

5 The present invention relates to novel Death Domain Containing Receptor (DR3 and DR3-V1) proteins which are members of the tumor necrosis factor (TNF) receptor family. In particular, isolated nucleic acid molecules are provided encoding the human DR3 and DR3-V1 proteins. DR3 and DR3-V1 polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR3 and DR3-V1 activity.

10 A97-10.WPD

99490-99499

10 30 50
CATGGGTGGGGTGGGGGCGCTGCTGGATTCTGCTCTGGTGGAGGGGAACTTGTGAGG

70 90 110
GGCTGGTAAGCGCCCCCTCCGAAGCCTGGTGTGTGCGCGGGGGGAAGGAAGTTAGTTTCC

130 150 170
TCTCCACCCATGGGCACCCCTTCTGCCCGGGGCTGGGAAGTGGGCTGCTCTGTGGGCAA

190 210 230
ATGCTGGGGCCTCTGAAATGGAGGAGACGCAGCAGGGAGAGGCCCCACGTGGGCAGCTGC
M E E T O O G E A P R G O L R

250 270 290
CGCGAGAGTCAGCAGCACCTGTCCCCCAGGCGCTCCTCCTGGTGTGCTGGGGGCCCGGG
G E S A A P V P O A L L L V L L G A R A

310 330 350
CCCAGGGCGGCACTCGTAGCCCCAGGTGTGACTGTGCCGGTGACTTCCACAAGAAGATTG
Q G G T R S P R C D C A G D F H K K I G

370 390 410
GTCTGTTTTGTGTCAGAGGCTGCCCAGCGGGCACTACCTGAAGGCCCTTGCACGGAGC
L F C C R G C P A G H Y L K A P C T E P

430 450 470
CCTGCGGCAACTCCACCTGCCTTGTGTGTGCCCAAGACACCTTCTTGGCCTGGGAGAACC
C G N S T C L V C P Q D T F L A W E N H

490 510 530
ACCATAATCTGAATGTGCCCGCTGCCAGGCCTGTGATGAGCAGGCCTCCAGGTGGCGC
H N S E C A R C Q A C D E Q A S Q V A L

550 570 590
TGGAGAACTGTTTTCAGCAGTGGCCGACACCCGCTGTGGCTGTAAGCCAGGCTGGTTTGTGG
E N C S A V A D T R C G C K P G W F V E

610 630 650
AGTGCCAGGTTCAGCCAATGTGTGTCAGCAGTTACCCCTTCTACTGCCAACCATGCCTAGACT
C Q V S Q C V S S S P F Y C Q P C L D C

670 690 710
GCGGGGCCCTGCACCGCCACACACGGCTACTCTGTTCGGCAGAGATACTGACTGTGGGA
G A L H R H T R L L C S R R D T D C G T

730 750 770
CCTGCCTGCCTGGCTTCTATGAACATGGCGATGGCTGCGTGTCTGCCCCACGAGCACCC
C L P G F Y E H G D G C V S C P T S T L

790 810 830
TGGGGAGCTGTCCAGAGCGCTGTGCCGCTGTCTGTGGCTGGAGGCAGATGTTCTGGGTCC
G S C P E R C A A V C G W R Q M F W V Q

850 870 890
AGGTGCTCCTGGCTGGCCTTGTGGTCCCCCTCTGCTTGGGGCCACCCTGACCTACACAT
V L L A G L V V P L L L G A T L T Y T Y

910 930 950
ACCGCCACTGCTGGCCTCACAAGCCCTGGTTACTGCAGATGAAGCTGGGATGGAGGCTC
R H C W P H K P L V T A D E A G M E A L

FIGURE 1A

Figure 1 consists of 12 histograms arranged in a 6x2 grid. The columns are labeled 'n=10' and 'n=20'. The rows are labeled 'k=1', 'k=2', 'k=3', 'k=4', 'k=5', and 'k=6'. Each histogram shows the frequency of the number of non-zero elements in the vector x . The x-axis for all histograms is 'Number of non-zero elements' and the y-axis is 'Frequency'. The distributions are generally centered around a value that increases with n and decreases with k .

FIGURE 1B

1 ATGGAGCAGC GGCCGCGGGG CTGCGCGGCG GTGGCGGCGG CGCTCCTCCT GGTGCTGCTG
M E Q R P R G C A A V A A A L L L V L L
61 GGGGCCCCGG CCCAGGGCGG CACTCGTAGC CCCAGGTGTG ACTGTGCCGG TGA CTTCAC
G A R A Q G G T R S P R C D C A G D F H
121 AAGAAGATTG GTCTGTTTTG TTGCAGAGGC TGCCAGCGG GGC ACTACCT GAAGGCCCCCT
K K I G L F C C R G C P A G H Y L K A P
181 TGCACGGAGC CCTGCGGCAA CTCCACCTGC CTTGTGTGTC CCCAAGACAC CTTCTTGGCC
C T E P C G N S T C L V C P Q D T F L A
241 TGGGAGAACC ACCATAATTC TGAATGTGCC CGCTGCCAGG CCTGTGATGA GCAGGCCTCC
W E N H H N S E C A R C Q A C D E Q A S
301 CAGGTGGCGC TGGAGAAGCTG TTCAGCAGTG GCCGACACCC GCTGTGGCTG TAAGCCAGGC
Q V A L E N C S A V A D T R C G C K P G
361 TGGTTTGTGG AGTGCCAGGT CAGCCAATGT GTCAGCAGTT CACCCTTCTA CTGCCAACCA
W F V E C Q V S Q C V S S S P F Y C Q P
421 TGCCTAGACT GCGGGGCCCC GCACCGCCAC ACACGGCTAC TCTGTTCCCG CAGAGATACT
C L D C G A L H R H T R L L C S R R D T
481 GACTGTGGGA CCTGCCTGCC TGGCTTCTAT GAACATGGCG ATGGCTGCGT GTCCTGCCCC
D C G T C L P G F Y E H G D G C V S C P
541 ACGAGCACCC TGGGGAGCTG TCCAGAGCGC TGTGCCGCTG TCTGTGGCTG GAGGCAGATG
T S T L G S C P E R C A A V C G W R Q M
601 TTCTGGGTCC AGGTGCTCCT GGCTGGCCTT GTGGTCCCC TCCTGCTTGG GGCCACCCTG
F W V Q V L L A G L V V P L L L G A T L
661 ACCTACACAT ACCGCCACTG CTGGCCTCAC AAGCCCCTGG TTACTGCAGA TGAAGCTGGG
T Y T Y R H C W P H K P L V T A D E A G
721 ATGGAGGCTC TGACCCACC ACCGGCCACC CATCTGTCAC CCTTGACAG CGCCACACC
M E A L T P P P A T H L S P L D S A H T
781 CTTCTAGCAC CTCCTGACAG CAGTGAGAAG ATCTGCACCG TCCAGTTGGT GGGTAACAGC
L L A P P D S S E K I C T V Q L V G N S
841 TGGACCCCTG GCTACCCCGA GACCCAGGAG GCGCTCTGCC CGCAGGTGAC ATGGTCCTGG
W T P G Y P E T Q E A L C P Q V T W S W
901 GACCAGTTGC CCAGCAGAGC TCTTGGCCCC GCTGCTGCGC CCACACTCTC GCCAGAGTCC
D Q L P S R A L G P A A A P T L S P E S
961 CCAGCCGGCT CGCCAGCCAT GATGCTGCAG CCGGGCCCGC AGCTCTACGA CGTGATGGAC
P A G S P A M M L Q P G P Q L Y D V M D
1021 GCGGTCCCAG CGCGGCGCTG GAAGGAGTTC GTGCGCACGC TGGGGCTGCG CGAGGCAGAG
A V P A R R W K E F V R T L G L R E A E
1081 ATCGAAGCCG TGGAGGTGGA GATCGGCCG TTCCGAGACC AGCAGTACGA GATGCTCAAG
I E A V E V E I G R F R D Q Q Y E M L K
1141 CGCTGGCGCC AGCAGCAGCC CGCGGGCCTT GGAGCCGTTT ACGCGGCCCT GGAGCGCATG
R W R Q Q Q P A G L G A V Y A A L E R M
1201 GGGCTGGACG GCTGCGTGGA AGACTTGCGC AGCCGCTGC AGCGCGGCC GTGA
G L D G C V E D L R S R L Q R G P

FIGURE 2

Consensus #1 M

DDCR M E E T Q Q G E A P R G Q L R G E S A A P V P Q A L L L V L 30
TNFR1 M G L S T V P D L L L P L V L L E L L V G I Y P S G V I G L 30
FAS M - L G I W T L L P L V L T S V A R L S S K S V N A Q V T D 29

Consensus #1 C

DDCR L G A R A Q G G T R S P R C D C A G D F H - - K K I G L F C 58
TNFR1 V P H L G D R E K R D S V C P Q G K Y I H - - P Q N N S I C 58
FAS I N S K G L E L R K T V T T V E T Q N L E G L H H D G Q F C 59

Consensus #1 . . . C . . G C C . . C

DDCR C R G C P A G H Y L K A P C T E P C G N S T C L V C P Q D T 88
TNFR1 C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S 88
FAS H K P C P P G E R K A R D C T V N G D E P D C V P C Q E G K 89

Consensus #1 H C . . C . . C C

DDCR F L A W E N H H N S E C A R C Q A C D E Q A S Q V A L E N C 118
TNFR1 F T A S E N H L R - H C L S C S K C R K E M G Q V E I S S C 117
FAS E Y T D K A H F S S K C R R C R L C D E G H G L E V E I N C 119

Consensus #1 T . C . C

DDCR S A V A D T R C G C K P G W F V E C - - - Q V S Q C V S S S 145
TNFR1 T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C - - - - 144
FAS T R T Q N T K C R C K P N F F Q N - - - - - - - - - - 137

Consensus #1 C

DDCR P F Y C Q P C L D C G A L H R H T R L L C S R R D T D C G T 175
TNFR1 - F N C S L C L N - G T V H - - - - L S C Q E K Q N T V C T 167
FAS - - - S T V C E H C D P - - - - - - - - - - C T K 148

Consensus #1 C . . G C C

DDCR C L P G F Y E H G D G C V S C P T S T L G - S C P E R C - - 203
TNFR1 C H A G F F L R E N E C V S C S N C K K S L E C T K L C L P 197
FAS C E H G I I - - - K E C - - - - - - - T L T S N T K C - - 166

Consensus #1 L

DDCR - - - - - A A V C G W R Q M F W V Q V L L A G L V V P L 225
TNFR1 Q I E N V K G T E D S G T T V L L P L V I F F G L C L L S L 227
FAS - - - - - K E E G S R S N L G W L C L L L - - L P I P L 186

Consensus #1

DDCR L L G G T L D L H I P P L L A H K P L V T A D E A G M E A L 255
TNFR1 L F I G - L M Y R Y Q R W K S K L Y S I V C G K S T P E K E 256
FAS I V - - - - - W V K R K E V - - - Q K T C R K H R 203

Consensus #1 G P

DDCR N P P P G T H L S P L D S A H T L L A P P D S S E K I C T V 285
TNFR1 G E L E G T T T K P L A P N P S F S P T P G F T P T L G F S 286
FAS K E N Q G S H E S P - - - - - - - - - - - - - 214

FIGURE 3

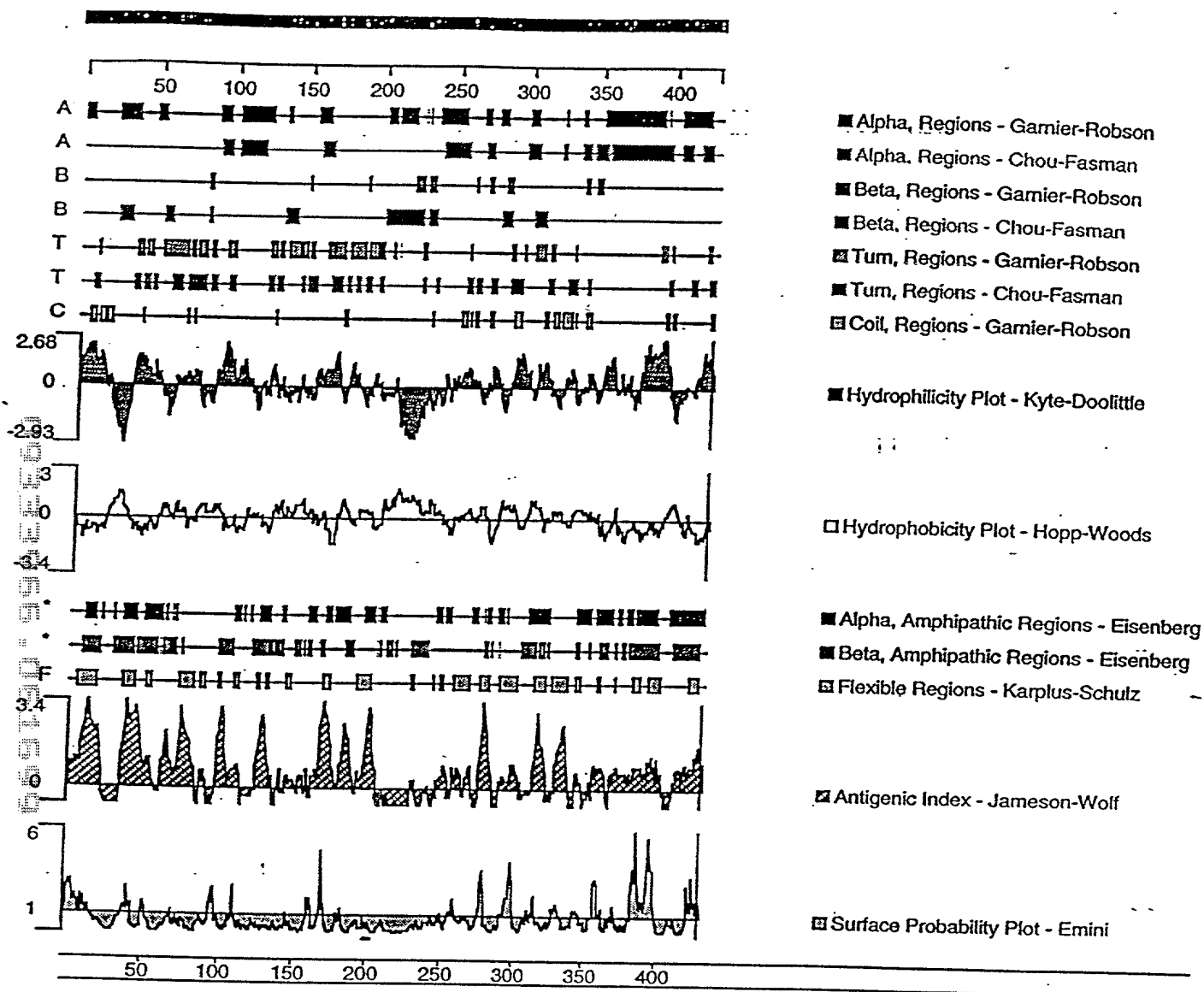


FIGURE 4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Yu et al.

Appl. No. To be assigned
(Div. of Appl. No. 08/815,469;
Filed: March 11, 1997)

Filed: Herewith

For: **Death Domain Containing
Receptors**

Art Unit: To be assigned

Examiner: To be assigned

Atty. Docket: 1488.0310005/EKS/SGW

Request to Open New Disk File

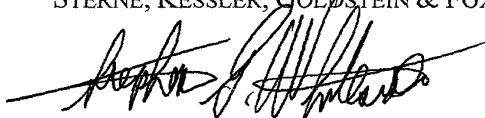
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants request that a new disk file be opened for the above-cited application. The Sequence Listing disk submitted on **March 11, 1997** in the parent, Application No. **08/815,469**, filed **March 11, 1997** contains the identical sequence information as that in the present application.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



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Combined Declaration and Power of Attorney for Patent Application

Docket Number: 1488.0310003

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter that is claimed and for which a patent is sought on the invention entitled **Death Domain Containing Receptors**, the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on **March 11, 1997**;
as United States Application Number or PCT International Application Number **08/815,469**; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)	
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional applications listed below.

<u>60/013,285</u>	<u>March 12, 1996</u>
(Application No.)	(Filing Date)
<u>60/028,711</u>	<u>October 17, 1996</u>
(Application No.)	(Filing Date)
<u>60/037,341</u>	<u>February 6, 1997</u>
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

<u>PCT/US96/03353</u>	<u>March 12, 1996</u>	<u>Pending</u>
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
<u>PCT/US96/16849</u>	<u>October 17, 1996</u>	<u>Pending</u>
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)

I hereby appoint the following attorneys(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Robert G. Sterne, Esq., Reg. No. 28,912; Edward J. Kessler, Esq., Reg. No. 25,688; Jorge A. Goldstein, Esq., Reg. No. 29,021; Samuel L. Fox, Esq., Reg. No. 30,353; David K.S. Cornwell, Esq., Reg. No. 31,944; Robert W. Esmond, Esq., Reg. No. 32,893; Tracy-Gene G. Durkin, Esq., Reg. No. 32,831; Michele A. Cimbala, Esq., Reg. No. 33,851; Michael B. Ray, Esq., Reg. No. 33,997; Robert E. Sokohl, Esq., Reg. No. 36,013; Eric K. Steffe, Esq., Registration No. 36,688; Andrea G. Reister, Esq., Registration No. 36,253; Robert H. Benson, Esq., Registration No. 30,446; Paul C. Kimball, Esq., Registration No. 34,610; and A. Anders Brookes, Esq., Registration No. 36,373.

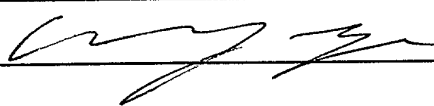
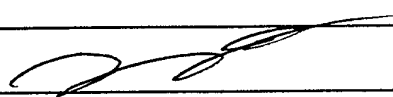
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Citizenship U.S.A.	
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Citizenship U.S.A.	
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